REMARKS

A Petition for Extension of Time is being concurrently filed with this Amendment. Thus, this Amendment is being timely filed.

Applicants respectfully request the Examiner to reconsider the present application in view of the foregoing amendments to the claims and the following remarks.

Status of the Claims

Claims 5, 6, 9, 11-18, and 20-24 are currently pending in the present application. The Office Action is final. Claims 15-16 have been withdrawn as directed to a non-elected invention. Claims 5, 6, 9 and 11 have been amended without prejudice or disclaimer. No new matter has been added by way of amendment. Claims 5 and 6 were amended to include "...and an optionally included tag sequence...," which has support at page 23, line 18 to page 27, line 15. Claim 9 has been amended into independent form by incorporating textual subject matter of claims 5 and 6. Support for amended claim 11 can be found on page 6, line 5 to page 7, line 11, and on page 23, line 18, to page 27, line 15 of the present specification. Thus no new matter has been added.

Based upon the above considerations, entry of the present Amendment is respectfully requested.

Objection to the Specification

With regards to line item 6 on page 3 of the Office Action dated April 18, 2008 (hereinafter "Office Action"), the Examiner indicates that the previous objection to the

specification (page 32, line 10) for reciting an amino acid sequences fused to GST without appropriate SEQ ID NO is maintained and requests an appropriate correction.

Applicants' representative thanks the Examiner for allowing Applicants' representative the opportunity to clarify this objection by telephone interview. Applicants' representative directed the Examiner's attention to the Amendment in Response to Non-Final Office Action, dated January 10, 2008, page 5 (third paragraph) to page 6 (first paragraph) where Applicants amended the paragraph to include SEQ ID numbers, of which item 6 of the Office Action was addressed.

Applicants respectfully request reconsideration and withdrawal of the present objection.

Claim Objections

With regards to line item 8 on page 3 of the Office Action, the Examiner indicates that claims 11-14 and 21-24 are objected to due to informalities.

Specifically, Claim 11 is objected to because claim 11 would be more clear if the claim recites "further having a tag sequence...." Applicants have amended claim 11, without prejudice or disclaimer to add "further" to the claim as suggested by the Examiner.

Since claim 11 was amended to include "further" as the Examiner suggested, the objection should be obviated as to claims 12-14 and 21-24.

Applicants respectfully request reconsideration and withdrawal of the present objection.

Rejection Under 35 U.S.C §112, Second Paragraph, Indefiniteness

Claims 5-6, 9, 11-14, 17-18 and 20-24 stand rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter, which Applicants regards as the invention.

The Examiner asserts that claim 5 and 6 (Claim 9, 11-14, 17-18 and 20-24 dependent therefrom) recites "a disintegrin-like and metalloprotease with thrombospondin type-1 motif, 13" and it is unclear to the Examiner what is encompassed by the limitation of "a disintegrin-like" and "metalloprotease with thrombospondin type-1 motif." Applicants respectfully traverse.

The name ADAMTS-13, "disintegrin-like and metalloprotease with thrombospondin type-1 motif, 13" is very well known in the art and is not a limitation. Applicants herein provide as an example that ADAMTS-13 is well known in the art, **Exhibit 1**, which is from the HUGO (Human Genome Organization) Gene Nomenclature Committee (hereinafter "HGNC"). HGNC through its website (www.genenames.org/data/hgnc_data.php?hgnc_id=1366), shows ADAMTS-13 is an acceptable term in the art to describe "a disintegrin-like and metalloprotease with thrombospondin type-1 motif, 13". Applicants respectfully request that the Examiner consider **Exhibit 1**, enclosed.

Since ADAMTS-13 is an accepted term within the art, it particularly points out and distinctly claims the subject matter.

With regards to claims 12, 13 and 17 (Claims 22 and 23 dependent therefrom) the Examiner asserts that the above claims recite "the mutant substrate polypeptide for ADAMTS-13 according to claim 11" and there is insufficient antecedent basis for this limitation in the claims. Applicants have amended claim 9, without prejudice or disclaimer, to independent form by

incorporating textual subject matter from claims 5 and 6 and additionally adding the full descriptive text for the term ADAMTS-13. Additionally claim 11 was amended, without prejudice or disclaimer, to additionally include the mutant substrate polypeptide for ADAMTS-13 according to claim 9. The above described amendments provide proper antecedent support for claims 12 and 13 (and therefore claims 22 and 23).

Applicants respectfully traverse with regards to claim 17, since claim 17 depends from claim 9 (and not from claim 11) and has proper antecedent basis.

Applicants respectfully request reconsideration and withdrawal of the present rejection.

Rejection Under 35 U.S.C § 112, First Paragraph, Written Description

Claims 9 and 20 stand rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement. The Examiner asserts that the claims contain subject matter not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor was in possession of the claimed invention at the time the application was filed. The Examiner asserts that the breadth of the claims encompass any amino acid sequence having 90% identity to SEQ ID NO: 4 (*i.e.*, the polypeptide of Claim 5) and having the specificity for ADAMTS-13, wherein the specificity is unclear based on the asserted 35 U.S.C. § 112, 2nd paragraph rejection. The Examiner further asserts that the prior art and the instant specification do not describe any polypeptide or any variant thereof within 90% and having any specificity sufficiently to represent the correlation between the structure of polypeptide (*e.g.*, any polypeptide having 90% identity to SEQ ID NO: 4 of Claim 5) and function of the specificity (that includes any specificity) for ADAMTS-13. Thus, the Examiner

suggests that the instant specification and the prior art cannot describe the structure of a very broad claimed genus and one skilled in the art would not be in possession of the full scope of claimed genus by the instant specification. Applicants respectfully traverse.

Claim 9 has not and does not refer to SEQ ID NO: 4 as the Examiner indicates. Further, the previous Office Action rejection the Examiner recited for this rejection also did not contain a reference to SEQ ID NO: 4. Applicants believe this is a transcribing error and that the Examiner meant to indicate SEQ ID NO: 1 within the rejection. Therefore, Applicants will address the Examiner's rejection with regards to SEQ ID NO: 1.

Applicants have amended claim 9, without prejudice or disclaimer, to independent form by incorporating textual subject matter from claims 5 and 6, additionally added the full descriptive text for the term ADAMTS-13 and removed the text regarding the retention of specificity for ADAMTS-13.

Applicants, respectfully submit that the presently amended claim 9 can be easily prepared and used by a skilled person in the art. **Exhibit 2 and 3 (Exhibit 2**: Kokame *et al.*, "*FRETS-VWF73, a First Fluorogenic Substrate for ADAMTS13 Assay*," British Journal of Haematology, vol.129, p.93-100 (2005) (Hereinafter "Kokame"), **Exhibit 3**: Wu *et al.*, "*Characterization of a Core Binding Site for ADAMTS-13 in the A2 Domain of Von Willebrand Factor*," PNAS, vol.103, no.49 p.18470-18474 (hereinafter "Wu")), which are enclosed for the Examiner's consideration, are described below.

Kokame describes that a mutant substrate FRETS-VWF73 was prepared and that this mutant substrate was successfully used as a substrate for ADAMTS-13 (*See* Kokame, Summary). Within FRETS-VWF73, the Q1599 residue was converted to a 2,3-diaminopropionic residue

(A2pr) modified with a 2-(N-methylamino)benzoyl group (Nma), and the N1610 residue was converted to A2pr modified with 2,4-dinitrophenylgroup (Dnp) (*See* Kokame, bridging paragraph from page 94 to 95). That is, two amino acids in the substrate claim 6 are substituted. Such a mutant substrate is included in the scope of claim 9.

Wu describes that mutant substrate polypeptides were prepared and that they were successfully used. Wu shows that the substrate H-A2-B containing an amino acid mutation Arg1659Ala, Glu1660Ala or Lys1668Ala was cleaved at a substantially same rate as the wild type (*See*, Wu, for example, Fig.5). Further, Wu shows that the Glu1655Ala mutation enhanced the rate of cleavage by 24% (See Wu, Fig.5). H-A2-B corresponds to the substrate polypeptide of claim 5 with the deletion of four amino acids (amino acids 1587-1590) and the substitution of one amino acid (*i.e.*, a total five amino acids were altered). H-A2-B also corresponds to the substrate polypeptide of claim 6 with the addition of five amino acids (amino acids 1591-1595) and the substitution of one amino acid (*i.e.*, a total of six amino acids were altered). That is, H-A2-B is included in the scope of claim 9. Moreover, it is reasonably recognized from the results of Wu that the mutant substrate polypeptide containing the combinations of any of the mutations Arg1659Ala, Glu1660Ala, Lys1668Ala and E1665A will also have the desired properties.

In addition, FRETS-VWF73 of Kokame is successfully used in several commercial kits for FRET-based assay for the measurement of ADAMTS-13 activity in human plasma. For example, FRETS-VWF73 is used in ACTIFLUOR™ ADAMTS13 Activity and ATS-13® (Please see **Exhibit 4**, ADAMTS-13 ADI Product catalog; assay information). The substrate polypeptide FRETS-VWF73 is also commercially available from PEPTIDE INSTITUTE, INC. and Peptides International Inc. (Please see **Exhibit 5**, web page from PEPTIDE INSTITUTE,

INC. and PEPNET Vol. 21, No.1 (2008) from Peptides International Inc.). Applicants respectfully request that the Examiner consider **Exhibits 4 and 5**.

The above exhibits show that the mutant substrate polypeptides of the invention are actually prepared and successfully used as a substrate for ADAMTS-13. Therefore, the claims contain subject matter that is described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor was in possession of the claimed invention at the time the application was filed.

Applicants respectfully request reconsideration and withdrawal of the present rejection.

Rejection Under 35 U.S.C §112, First Paragraph, Enablement

Claims 9 and 20 stand rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the enablement requirement. The Examiner suggests that the claims contain subject matter not described in the specification in such a way as to enable one skilled in the art to make or use the invention commensurate within the scope of the claims.

The Examiner asserts that the specification, while enabling for a substrate polypeptide for an isolated polypeptide of SEQ ID NO: 4; does not reasonably provide enablement for an amino acid having 90% identity to SEQ ID NO: 4 and retains "the specificity for ADAMTS-13", wherein the specificity includes any specificity. Applicants respectfully traverse.

Claim 9 has not and does not refer to SEQ ID NO: 4 as the Examiner indicates. Further, the previous Office Action rejection the Examiner recited for this rejection also did not contain a reference to SEQ ID NO: 4. Applicants believe this is a transcribing error and that the Examiner

meant to indicate SEQ ID NO: 1 within the rejection. Therefore, Applicants will address the Examiner's rejection with regards to SEQ ID NO: 1.

Although Applicants disagree, in order to further prosecution, they have amended claim 9, without prejudice or disclaimer, to independent form by incorporating textual subject matter from claims 5 and 6, and removed the text "which retains the specificity for ADAMTS-13" so as to further define the claim.

Applicants respectfully request reconsideration, and subsequent withdrawal of the present rejection.

In view of the above remarks, Applicants believe the pending application is in condition for allowance.

CONCLUSION

A full and complete response has been made to all issues as cited in the Office Action. Applicants have taken substantial steps in efforts to advance prosecution of the present application. Thus, Applicants respectfully request that a timely Notice of Allowance issue for the present case.

In view of the above remarks, it is believed that claims are allowable.

Should there be any outstanding matters within the present application that need to be resolved, the Examiner is respectfully requested to contact Paul D. Pyla, Reg. No. 59,228, at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37.C.F.R. §§1.16 or 1.14; particularly, extension of time fees.

Dated: AUG 11 2008

Respectfully submitted,

By
Gerald M. Murphy, Jr.
Registration No.: 28,977
BIRCH, STEWART, KOLASCH & BIRCH, LLP
8110 Gatehouse Road
Suite 100 East
P.O. Box 747
Falls Church, Virginia 22040-0747
(703) 205-8000
Attorney for Applicants

Attachments:

Exhibit 1: HGNC (HUGO (Human Genome Organisation) Gene Nomenclature Committee) website (http://www.genenames.org/data/hgnc_data.php?hgnc_id=1366), shows ADAMTS-13 as an acceptable term in the art to describe "a disintegrin-like and metalloprotease with thrombospondin type-1 motif, 13".

Exhibit 2: Kokame *et al.*, "*FRETS-VWF73, a First Fluorogenic Substrate for ADAMTS13 Assay*," British Journal of Haematology, vol.129, p.93-100 (2005).

Exhibit 3: Wu *et al.*, "*Characterization of a Core Binding Site for ADAMTS-13 in the A2 Domain of Von Willebrand Factor*," PNAS, vol.103, no.49 p.18470-18474 (2006).

Exhibit 4: ADAMTS-13 ADI Product catalog; assay information.

Exhibit 5: Web page from PEPTIDE INSTITUTE, INC. and PEPNET Vol. 21, No.1 (2008) from Peptides International Inc.

Symbol Report: ADAMTS13

EXHIBIT 1

Giving unique and meaningful names to every human gene

Core Data		Database Links		
Approved Symbol +	ADAMTS13	**Accession Numbers +**		
Approved Name +	ADAM metallopeptidase with thrombospondin type 1 motif, 13	AJ011374	GenBank	UCSC Browser
HGNC ID +	HGNC:1366	**Pubmed IDs +**		
Status +	Approved	11557746 11535495	PMID	
Chromosome +	9q34	**VEGA IDs +**		
Previous Symbols +	C9orf8	OTTHUMG00000020876	VEGA GeneView	
Previous Names +	"a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 13"	**Ensembl ID (mapped data supplied by Ensembl) +**		
Aliases +	VWFCP	ENSG00000160323	Ensembl GeneView	
Name Aliases +		**Entrez Gene ID (mapped data supplied by NCBI) +**		
		11093	Gene	Map Viewer
Gene Symbol Links		**RefSeq (mapped data supplied by NCBI) +**		
GENATLAS GeneCards GeneClinics/GeneTests GoPubmed		NM_139026	GenBank	UCSC Browser / UCSC Index
HCOP H-InvDB Treefam		**OMIM ID (mapped data supplied by NCBI) +**		
		604134	OMIM	
		UCSC ID (mapped data supplied by UCSC) +		
		uc004cdv.1	UCSC Index	
		UniProt ID (mapped data supplied by UniProt) +		
		Q76LX8	SwissProt	UniProt
		Specialist Database Links +		
		MEROPS:M12.241		

See Column definitions for descriptions of the various data fields. Also see our custom downloads page for bulk access to our data

NHGRI

wellcome trust

The work of the HGNC is supported by NHGRI grant P41 HG003345, the UK Medical Research Council and the Wellcome Trust

EMBL-EBI

EXHIBIT 2

FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay

Koichi Kokame,[1] Yuko Nobe,[1] Yoshihiro Kokubo,[2] Akira Okayama[2] and Toshiyuki Miyata[1]

[1]*National Cardiovascular Centre Research Institute, and* [2]*Department of Preventive Cardiology, National Cardiovascular Centre, Suita, Osaka, Japan*

Received 17 November 2004; accepted for publication 27 January 2005

Correspondence: Koichi Kokame, National Cardiovascular Centre Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail: kame@ri.ncvc.go.jp

Summary

A plasma metalloprotease, ADAMTS13, cleaves von Willebrand factor (VWF) multimers and downregulates their activity in platelet aggregation. Functional ADAMTS13 deficiency leads to the accumulation of hyperactive large VWF multimers, inducing a life-threatening disease, thrombotic thrombocytopenic purpura (TTP). Although measuring ADAMTS13 activity is important in TTP diagnosis, existing methods require time and skill. Here, we report a fluorescence resonance energy transfer (FRET) assay for ADAMTS13 activity. We developed a synthetic 73-amino-acid peptide, FRETS-VWF73. Cleavage of this substrate between two modified residues relieves the fluorescence quenching in the intact peptide. Incubation of FRETS-VWF73 with normal human plasma quantitatively increased fluorescence over time, while ADAMTS13-deficient plasma had no effect. Quantitative analysis could be achieved within a 1-h period using a 96-well format in commercial plate readers with common filters. The FRETS-VWF73 assay will be useful for the characterization of thrombotic microangiopathies like TTP and may clarify the importance of ADAMTS13 activity as a predictive marker for various thrombotic diseases.

Keywords: ADAMTS13, von Willebrand factor, platelet, thrombotic thrombocytopenic purpura, fluorescence resonance energy transfer.

Thrombotic thrombocytopenic purpura (TTP), a syndrome characterized by thrombocytopenia and microangiopathic haemolytic anaemia, is often associated with neurological dysfunction, renal failure and fever (Moschcowitz, 1924; Moake *et al*, 1982). Although most patients with TTP experience these crises idiopathically throughout adulthood, some patients present with neonatal onset and frequent relapses, also called Upshaw–Schulman syndrome (USS). Recent genetic studies have revealed that the majority of USS patients are homozygous or compound heterozygous for a critical mutation of the *ADAMTS13* gene (Levy *et al*, 2001; Kokame *et al*, 2002; Antoine *et al*, 2003; Assink *et al*, 2003; Savasan *et al*, 2003; Schneppenheim *et al*, 2003; Matsumoto *et al*, 2004; Pimanda *et al*, 2004). *ADAMTS13* encodes a plasma metalloprotease of the ADAMTS family (Levy *et al*, 2001; Soejima *et al*, 2001; Zheng *et al*, 2001; Plaimauer *et al*, 2002; Banno *et al*, 2004). Many patients with acquired TTP possess inhibitory auto-antibodies against ADAMTS13 (Furlan *et al*, 1998; Tsai & Lian, 1998). As the clinical characteristics of TTP are similar to those of other microangiopathic haemolytic anaemias, such as haemolytic uremic syndrome (HUS) and disseminated intravascular coagulation (DIC), an assay measuring ADAMTS13 activity would be an useful tool for appropriate diagnosis and treatment of TTP.

ADAMTS13 cleaves the peptidyl bond between Y1605 and M1606 in the A2 domain of von Willebrand factor (VWF) (Dent *et al*, 1990; Tsai *et al*, 1994; Furlan *et al*, 1996; Tsai, 1996), which circulates in plasma as large multimeric forms, ranging in size from 500 to 20 000 kDa. Functional ADAMTS13 deficiency can lead to the accumulation of large, hyperactive VWF multimers. A method to measure VWF-cleavage activity of ADAMTS13 was originally developed by Furlan *et al* (1996) and Tsai (1996), in which purified human VWF multimers were incubated with plasma in the presence of either urea or guanidine. The reaction products were separated by sodium dodecyl sulphate (SDS)-agarose (Furlan *et al*, 1996) or SDS-polyacrylamide (Tsai, 1996) gel electrophoresis, followed by Western blotting analysis with anti-VWF antibodies. Although these methods have significantly increased our understanding of the role of ADAMTS13 in TTP pathogenesis, they are not widely used at the clinical level because of technical complications.

Several groups have attempted to develop more simple and rapid diagnostic procedures for clinical use, including a

  doi:10.1111/j.1365-2141.2005.05420.x

collagen-binding assay (Gerritsen *et al*, 1999), an immunoradiometric assay using two site-directed VWF antibodies (Obert *et al*, 1999) and a ristocetin-cofactor assay (Böhm *et al*, 2002). Multicentre comparison studies of these different assays showed varied performance but supported the usefulness of the ADAMTS13 assay for TTP diagnosis (Studt *et al*, 2003; Tripodi *et al*, 2004). These assays, however, still demand complicated procedures and highly specialized materials. Therefore, a more rapid, reliable and convenient method of measuring VWF activity is eagerly awaited.

As chromogenic substrate assays are used in the clinical measurement of protease activities, initial studies were sought to identify a short oligopeptide that can be specifically cleaved by ADAMTS13 (Furlan & Lämmle, 2002). As these attempts have systematically failed, the cleavage at Y1605–M1606 of VWF probably depends on both the specific residues in the vicinity of the scissile bond and more remote sequences. Recently, we have succeeded in creating a recombinant substrate encompassing the shortest region of VWF that serves as a specific substrate for ADAMTS13 (Kokame *et al*, 2004). The peptide substrate, designated VWF73, contains 73-amino-acid residues of VWF from D1596 to R1668. In this study, we have chemically modified VWF73 to facilitate the quantitative measurement of ADAMTS13 activity in a single-step procedure.

Materials and methods

Materials

The fluorogenic substrate, FRETS-VWF73, was chemically synthesized by Thermo Electron GmbH (Sedanstrasse, Ulm, Germany) and the Peptide Institute, Inc. (Osaka, Japan). It was dissolved in 25% dimethyl sulphoxide/water to prepare the 100-μmol/l stock solution. Human plasma was obtained by centrifugation from whole blood that was treated with a 1/10 volume of 3·8% sodium citrate as an anti-coagulant. A protease inhibitor cocktail (Sigma, St Louis, MO, USA) used in the cleavage experiments contained 1 mmol/l 4-(2-aminoethyl) benzenesulphonyl fluoride, 15 μmol/l pepstatin A, 14 μmol/l *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 36 μmol/l bestatin, 21 μmol/l leupeptin and 0·8 μmol/l aprotinin at a final concentration.

Fluorescent assay to measure the ADAMTS13 activity

Pooled human plasma (a range of 0–8 μl as a standard), or 4 μl of each test plasma, was diluted in 100 μl of assay buffer (5 mmol/l Bis–Tris, 25 mmol/l $CaCl_2$, 0·005% Tween-20, pH 6·0) in a 96-well white plate (Sumitomo Bakelite, Tokyo, Japan). Then, 100 μl of 4 μmol/l FRETS-VWF73 in the assay buffer was added to each well. Fluorescence was measured at 30°C in a Wallac 1420 ARVO multilabel counter (PerkinElmer Japan, Yokohama, Japan) equipped with a 340-nm excitation filter and a 450-nm emission filter. Fluorescence was measured every 5 min. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 to 60 min using the PRISM software (GraphPad Software, San Diego, CA, USA).

Preparation of recombinant ADAMTS13 (rADAMTS13)

HeLa cells were cultured in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, NM, USA) supplemented with 10% fetal bovine serum in humidified air with 5% CO_2 at 37°C. To produce rADAMTS13, the human ADAMTS13-expression plasmid was transfected into the subconfluent cells using FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA), as described previously (Kokame *et al*, 2002; Matsumoto *et al*, 2004). Following a 4-h incubation, the culture medium was replaced with serum-free OPTI-MEM I medium (Invitrogen) and the culture was incubated for 44 h. The medium was collected and concentrated to one-eighth the original volume using Centricon YM-30 (Millipore, Billerica, MA, USA). As a negative control, a series of operations was performed in parallel as for the untransfected cells.

Subject population

The Suita study participants were arbitrarily selected from the municipality population registry of Suita city, stratified by gender and 10-year age groups. The basic sampling of the population started in 1989 with a cohort study base (Mannami *et al*, 1997). In the present study, 100 consecutive samples were selected from this population as a control group. This study was approved by the ethical committee on human research of the National Cardiovascular Centre. Written informed consent was obtained from all subjects prior to testing.

Results

Design of the fluorogenic substrate for ADAMTS13

To utilize fluorescence resonance energy transfer (FRET) to measure ADAMTS13 activity, we chemically synthesized a fluorogenic peptide, FRETS-VWF73 (Fig. 1), containing the 73-amino acids from D1596 to R1668 of VWF. Within this peptide, the Q1599 residue at the P7 position was converted to a 2,3-diaminopropionic residue (A2pr) modified with a 2-(*N*-

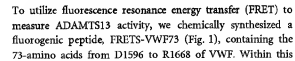


Fig 1. Structure of FRETS-VWF73. Within the 73-amino-acid peptide sequence, corresponding to the region from D1596 to R1668 of von Willebrand factor (VWF), Q1599 and N1610 were substituted with A2pr(Nma) and A2pr(Dnp) respectively. The arrowhead indicates the site cleaved by ADAMTS13.

methylamino)benzoyl group (Nma). The N1610 residue of the P5′ position was converted to A2pr modified with a 2,4-dinitrophenyl group (Dnp). When the Nma group is excited at 340 nm, fluorescence resonance energy is transferred to the neighbouring quencher, Dnp. If the bond between Y1605 and M1606 is cleaved, the energy transfer quenching the fluorescence does not occur, allowing the emission of fluorescence at 440 nm from Nma.

Cleavage of FRETS-VWF73 by plasma ADAMTS13

To explore the cleavage activity present in plasma, FRETS-VWF73 was incubated with normal human plasma in a fluorescent plate reader. Emission at 450 nm increased with time, indicating that FRETS-VWF73 was cleaved between the two A2pr residues by a plasma component (Fig. 2A). The increase of fluorescence was not inhibited by the addition of a protease inhibitor cocktail (mixed inhibitors effective against a broad range of serine proteases, cysteine proteases, aminopeptidases and acid proteases), but was completely inhibited by a divalent cation chelator (EDTA), suggesting that cleavage was mediated by the plasma metalloprotease, ADAMTS13, with minimal contribution of other plasma proteases. In fact, neither thrombin nor plasmin (5 μg/ml each, Sigma) increased fluorescence of FRETS-VWF73 (data not shown). The incubation of FRETS-VWF73 with plasma from an ADAMTS13-deficient patient showed no increase of fluorescence (Fig. 2A). The addition of ADAMTS13-deficient plasma to the normal plasma did not interfere with the cleavage of FRETS-VWF73 by the normal plasma (data not shown).

To verify further the cleavage by ADAMTS13, the substrate was incubated with the conditioned medium of cultured HeLa cells (Fig. 2B). Incubation with the medium of ADAMTS13-transfected cells showed the time-dependent increase of fluorescence, whereas the incubation with the medium of untransfected cells did not. All these data supported the conclusion that ADAMTS13 specifically cleaved FRETS-VWF73.

Plasma-dose dependency

FRETS-VWF73 cleavage was quantitatively dependent on plasma dosage (Fig. 3). We monitored fluorescence increase in the presence of variable volumes of normal plasma to the reaction mixture. The fluorescence over time increased with increasing plasma in a dose-dependent manner (Fig. 3A). To compensate for any differences in background fluorescence derived from plasma itself and to calculate the initial reaction rate, we estimated the slopes of the fluorescence over time using time points 0 and 60 min from a linear regression. These slopes (reaction rates) were then plotted against the plasma dosage (Fig. 3B). The data points fitted to a non-linear regression, indicating that ADAMTS13 activity in sample plasma could be estimated from the fluorescence reaction rate.

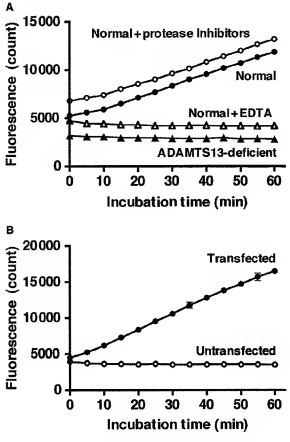


Fig 2. Cleavage of FRETS-VWF73 by ADAMTS13. (A) Fluorescence changes in FRETS-VWF73 during incubation with plasma. FRETS-VWF73 was incubated with normal plasma from a single donor in the absence (closed circles) or presence of either protease inhibitors (open circles) or EDTA (open triangles). The substrate was also incubated with ADAMTS13-deficient plasma from a congenital thrombotic thrombocytopenic purpura patient (closed triangles). Fluorescent emission at 450 nm was measured at the indicated times. A representative of three repetitive experiments is shown. (B) Fluorescence changes in FRETS-VWF73 during incubation with recombinant ADAMTS13 (rADAMTS13). FRETS-VWF73 was incubated with the conditioned medium of HeLa cells transfected with (closed circles) or without (open circles) ADAMTS13-expression plasmid DNA. Values shown are the mean and SD ($n = 3$).

Optimization of the FRETS-VWF73 assay

We next optimized reaction conditions to increase both the sensitivity and rapidity of measurement (Fig. 4). As ADAMTS13 requires divalent metal ions for proteolytic activity, we monitored the cleavage of FRETS-VWF73 by plasma in the presence of various metal ions (Fig. 4A). Ca^{2+} and Ba^{2+} ions were the most favourable for the reaction, although Mg^{2+} and Zn^{2+} also enhanced ADAMTS13 activity. In contrast, Mn^{2+} and Ni^{2+} could not activate the reaction, consistent with previous reports (Furlan *et al*, 1996; Tsai, 1996). Testing of various Ca^{2+} ion concentrations revealed that a range of

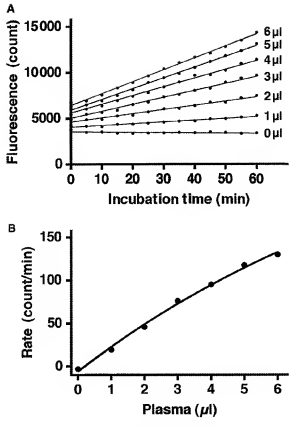


Fig 3. Plasma-dose dependency of FRETS-VWF73 cleavage. (A) Fluorescence was measured at 5-min intervals after the addition of FRETS-VWF73 to 0–6 µl normal plasma. (B) The reaction rates of time points 0 and 60 min were plotted against plasma dosage. Values were fit to a non-linear regression curve.

10–50 mmol/l Ca^{2+} was optimal for the reaction (Fig. 4B). We also examined the effect of differing NaCl concentrations, determining that lower concentrations provided more rapid cleavage (Fig. 4C), as seen in previous reports (Furlan *et al*, 1996; Kokame *et al*, 2004). The pH optimum for the FRETS-VWF73 assay was approximately 6·0 (Fig. 4D), which differed from previous studies reporting an optimal pH of 8·0–10·0 for the cleavage reaction (Furlan *et al*, 1996). This inconsistency may be a result of different reaction conditions, such as the presence or absence of denaturants. In addition, substitution of Q1599 and N1610 to A2pr(Nma) and A2pr(Dnp), respectively,

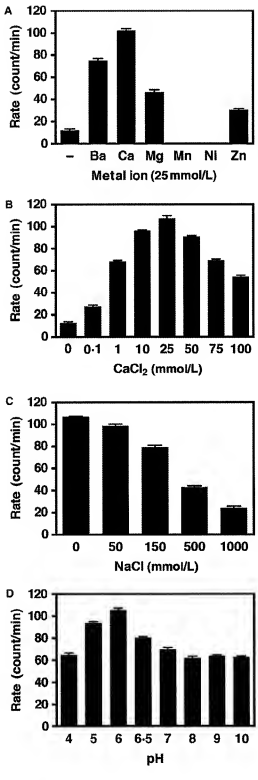


Fig 4. Optimization of the FRETS-VWF73 assay. (A) To determine metal ion dependency, FRETS-VWF73 was incubated with normal plasma from a single donor in the presence of the indicated divalent ions. (B) To measure Ca^{2+}-concentration dependency, FRETS-VWF73 was incubated with plasma in the presence of 0–100 mmol/l $CaCl_2$. (C) Ion-strength dependency was determined by incubating FRETS-VWF73 with plasma in the presence of 0–1000 mmol/l NaCl. (D) To measure pH dependency, FRETS-VWF73 was incubated with plasma in the indicated pH buffer. The reaction rates of time points 0 and 60 min are shown with the mean and SD ($n = 3$).

may affect the cleavage pH dependency. Alternatively, pH dependency of the assay might be affected not only by the cleavage efficiency, but also fluorescence emission, because most fluorescence reactions are highly pH dependent. Regardless, these data indicated that the FRETS-VWF73 assay was most efficient in the reaction buffer containing 5 mmol/l Bis–Tris, 25 mmol/l $CaCl_2$ and 0·005% Tween 20 at pH 6·0.

Reproducibility

We examined inter-run reproducibility of the FRETS-VWF73 assay. Plasma-dose dependency in the optimized condition was observed independently seven times. Each regression curve corresponded well with the other curves, indicating that the assay was obviously reproducible (data not shown). The relative ADAMTS13 activities of three different plasma samples were also measured independently seven times, where the activity of pooled plasma was normalized as 100%. The mean ± standard deviation (SD) values of the three samples were 113·9 ± 2·4, 62·5 ± 2·1 and 22·3 ± 1·4% ($n = 7$), respectively, indicating that the inter-assay variation was significantly small. The coefficients of variation of the three samples were 2·1, 3·4 and 6·3% ($n = 7$) respectively.

Plasma ADAMTS13 activity of patients and healthy individuals

To evaluate the FRETS-VWF73 assay for potential clinical use, we measured the relative ADAMTS13 activity in 78 plasma samples from various patients and 100 healthy individuals (Fig. 5A). The relative activities were estimated from the activity of pooled plasma prepared from all the 100 healthy individuals (66·6 ± 11·7 years old). Plasma samples from congenital TTP patients, homozygotes or compound heterozygotes of critical *ADAMTS13* mutations (Kokame *et al*, 2002; Matsumoto *et al*, 2004), all exhibited very low (<1%) or undetectable activities. The majority (33 samples) of plasma samples obtained from 41 patients with idiopathic TTP also showed low (<5%) or undetectable activities. The most possible explanation would be a deficiency of plasma ADAMTS13 level or generation of auto-antibodies against ADAMTS13, although there may be some other factor, such as auto-antibodies, that bind to the substrate and protect it from being cleaved. In contrast, plasma from parents or siblings of

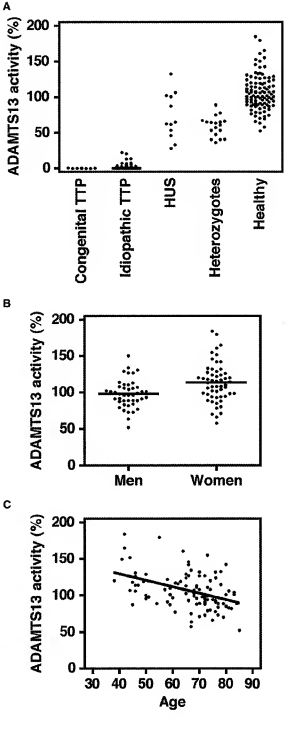


Fig 5. ADAMTS13 activity in plasma from patients and healthy individuals. (A) Relative ADAMTS13 activity was measured using the FRETS-VWF73 assay. FRETS-VWF73 was incubated with 4 μl of plasma from seven congenital thrombotic thrombocytopenic purpura (TTP) patients, 41 idiopathic TTP patients, 12 haemolytic uremic syndrome patients and 18 heterozygotes of ADAMTS13 with critical mutations. Plasma samples from randomly selected 100 healthy individuals were also examined. The relative ADAMTS13 activities were estimated from the standard curve, which was drawn up on the basis of the reaction rates of the pooled plasma prepared from all the 100 healthy individuals. (B) Association of ADAMTS13 activity with gender. Plasma ADAMTS13 activity in the healthy individuals was plotted by gender (45 men and 55 women). Bars indicate the means. Unpaired *t*-test identified significant differences between men and women ($P = 0·0016$). (C) Association of ADAMTS13 activity with age. The plasma ADAMTS13 activity correlated significantly with age ($P < 0·0001$). A line of best fit is indicated.

congenital TTP patients, heterozygotes of *ADAMTS13* mutations, exhibited on average approximately half the activity (59·0 ± 14·4%) of healthy individuals, while the plasma of patients with HUS showed substantial activity (73·2 ± 32·3%). Thus, the FRETS-VWF73 assay can be used to measure ADAMTS13 activity for TTP diagnosis in clinical samples.

Association of ADAMTS13 activity with gender and age

The measured ADAMTS13 activities of plasma samples from 100 healthy individuals (45 men aged 67·4 ± 11·5 years old and 55 women aged 64·9 ± 11·8 years old) were plotted according to gender (Fig. 5B). Comparison of the ADAMTS13 activities between men (97·9 ± 19·2%) and women (113·5 ± 27·1%) using the unpaired *t*-test demonstrated a significant difference between these groups ($P = 0{\cdot}0016$), suggesting that the ADAMTS13 activity of women should be significantly higher than that of men. Examination of the effect of age on ADAMTS13 activities using Spearman's rank correlation revealed a significant correlation ($r = -0{\cdot}396$, $P < 0{\cdot}0001$) (Fig. 5C). The slopes of best fit in linear regression analysis were −0·894 ± 0·196, with R^2 values of 0·175 ($P < 0{\cdot}0001$), suggesting that plasma ADAMTS13 activity should decrease with advancing age, at least after the early 40s.

Discussion

The Y1605–M1606 bond is inaccessible in native VWF and made sensitive to ADAMTS13 by denaturation and shear force. Structural modelling has suggested that the bond is buried in the core β-sheet of the VWF A2 domain (Jenkins *et al*, 1998; Sutherland *et al*, 2004). This partially explains the requirement for denaturants or shear force in the hydrolysis of the Y1605–M1606 bond by ADAMTS13. VWF73, corresponding to the C-terminal two-fifths of the A2 domain, can be efficiently cleaved by ADAMTS13 in the absence of denaturants and shear force (Kokame *et al*, 2004), suggesting that the N-terminal three-fifths of the A2 domain may prevent ADAMTS13 from accessing the cleavage site. A recent study indicated that the VWF A1 domain inhibits cleavage of the A2 domain by ADAMTS13; binding of platelet glycoprotein Ibα to the A1 domain appears to relieve the inhibition (Nishio *et al*, 2004). As VWF73 is a relatively small substrate, cleavage is less likely to be affected by other molecules. Therefore, VWF73 is an appropriate core for the convenient single-step fluorogenic assay for ADAMTS13 activity developed in this study.

Being a chemically modified version of VWF73 containing A2pr(Nma) and A2pr(Dnp), FRETS-VWF73 was a good substrate for ADAMTS13 cleavage, suggesting that Q1599 at the P7 position and N1610 at the P5′ position are not essential for the cleavage. We also examined the substitution of N1602 at the P4 position to A2pr(Nma). Although the peptide could be cleaved by plasma ADAMTS13, the efficiency was lower than that of the original FRETS-VWF73 (data not shown). The shorter distance of the modified residue from the cleavage site may interfere with efficient cleavage by ADAMTS13.

Enzymatic studies of ADAMTS13 will progress using FRETS-VWF73 as a model substrate in the future. The previously established substrate, purified plasma VWF, is comprised of non-uniform multimers with multiple cleavage sites. In contrast, FRETS-VWF73 is a monomeric molecule with a single cleavage site, facilitating the determination of cleavage kinetic parameters. No denaturants are required for the reaction, making this assay more closely reflect the physiological conditions. Although the optimal cleavage of FRETS-VWF73 still requires a hypotonic environment, isotonic solution gives approximately 80% of the activity observed in NaCl-free conditions (Fig. 3C) for kinetic analyses. VWF73, however, is not suitable for studying the functions of the other VWF domains, such as A1 and A3.

The greatest impact of the FRETS-VWF73 assay will be as a potential clinical diagnostic test. Unlike previous assays, the assay is a simple procedure, requiring no special reagents or equipment except a fluorescence spectrophotometer. These advantages may popularize ADAMTS13-activity measurement at the clinical level. The best possible application will be the appropriate diagnosis of TTP. The FRETS-VWF73 assay could be useful also for selecting curative plasma before administration to patients, as ADAMTS13 activity in the general population varies widely (Fig. 5). The selection of high-titre plasma may improve the responses of patients to plasma infusion or exchange treatment.

The relationship between ADAMTS13 deficiency and TTP is more complicated than originally thought (George & Vesely, 2004; Zheng *et al*, 2004); the problem may be because of symptomatical and pathological variety and diagnostic criteria of TTP. Not all patients with TTP present the classical five features of disease, thrombocytopenia, microangiopathic haemolytic anaemia, neurological dysfunction, renal failure and fever. Although severe ADAMTS13 deficiency is observed in most patients with idiopathic TTP without pre-existing medical conditions (Furlan *et al*, 1998; Tsai & Lian, 1998), the association between ADAMTS13 deficiency and TTP is unclear in less highly selected patient groups (Veyradier *et al*, 2001; Vesely *et al*, 2003). ADAMTS13 measurement cannot be used to predict exactly response to plasma exchange in patients that are clinically diagnosed with TTP (Vesely *et al*, 2003). An accurate ADAMTS13 assay may help to categorize TTP patients into subgroups and help establish objective diagnostic criteria.

What should be the cut-off value of ADAMTS13 activity for the diagnosis of TTP or ADAMTS13 deficiency? The present and previous studies (Mannucci *et al*, 2001; Veyradier *et al*, 2001; Böhm *et al*, 2002) showed a wide distribution of the ADAMTS13 activity in the healthy population. Further, we showed that the ADAMTS13 activity was associated with gender and age. As we used pooled plasma that was derived from relatively older individuals as a standard, the apparent ADAMTS13 activity of patient plasma may be over estimated

in the present study. To determine the universally applicable cut-off value, the definition of standard plasma will be of primary importance. The availability of purified or recombinant ADAMTS13 may help the standardization of ADAMTS13 assay. The gender- and age-oriented distribution of ADAMTS13 activity will need to be determined in the general population. Although the FRETS-VWF73 assay detected significant activity in some idiopathic TTP patients, the value was evidently lower than the lowest activity of 100 healthy individuals (Fig. 5A). Therefore, the cut-off value (for instance, the mean −2 SD % of normal activity) may have to be determined considering gender and age. The FRETS-VWF73 assay, suitable for high-throughput measurement, would accelerate such a population study.

Acknowledgements

We thank Dr Masanori Matsumoto and Dr Yoshihiro Fujimura for patient plasma, Dr Kenji Soejima for ADAMTS13-expression plasmid, Dr Masahiko Tsunemi for critical discussion and the members of the Satsuki–Junyukai for attending the project. This work was supported in part by grants-in-aid from the Ministry of Health, Labour and Welfare of Japan; the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Program for Promotion of Fundamental Studies in Health Science of the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan; and Mitsubishi Pharma Research Foundation.

References

Antoine, G., Zimmermann, K., Plaimauer, B., Grillowitzer, M., Studt, J.D., Lämmle, B. & Scheiflinger, F. (2003) *ADAMTS13* gene defects in two brothers with constitutional thrombotic thrombocytopenic purpura and normalization of von Willebrand factor-cleaving protease activity by recombinant human ADAMTS13. *British Journal of Haematology*, **120**, 821–824.

Asselta, R., Schiphorst, R., Allford, S., Karpman, D., Etzioni, A., Richard, B., van de Kar, N., Monnens, L. & van den Heuvel, L. (2003) Mutation analysis and clinical implications of von Willebrand factor-cleaving protease deficiency. *Kidney International*, **63**, 1995–1999.

Banno, F., Kaminaka, K., Soejima, K., Kokame, K. & Miyata, T. (2004) Identification of strain-specific variants of mouse *Adamts13* gene encoding von Willebrand factor-cleaving protease. *Journal of Biological Chemistry*, **279**, 30896–30903.

Böhm, M., Vigh, T. & Scharrer, I. (2002) Evaluation and clinical application of a new method for measuring activity of von Willebrand factor-cleaving metalloprotease (ADAMTS13). *Annals of Hematology*, **81**, 430–435.

Dent, J.A., Berkowitz, S.D., Ware, J., Kasper, C.K. & Ruggeri, Z.M. (1990) Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 6306–6310.

Furlan, M. & Lämmle, B. (2002) Assays of von Willebrand factor-cleaving protease: a test for diagnosis of familial and acquired thrombotic thrombocytopenic purpura. *Seminars in Thrombosis and Hemostasis*, **28**, 167–172.

Furlan, M., Robles, R. & Lämmle, B. (1996) Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood*, **87**, 4223–4234.

Furlan, M., Robles, R., Galbusera, M., Remuzzi, G., Kyrle, P.A., Brenner, B., Krause, M., Scharrer, I., Aumann, V., Mittler, U., Solenthaler, M. & Lämmle, B. (1998) von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic uremic syndrome. *New England Journal of Medicine*, **339**, 1578–1584.

George, J.N. & Vesely, S.K. (2004) ADAMTS13 and TTP: the clot thickens. *Blood*, **103**, 3997–3998.

Gerritsen, H.E., Turecek, P.L., Schwarz, H.P., Lämmle, B. & Furlan, M. (1999) Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF: a tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). *Thrombosis and Haemostasis*, **82**, 1386–1389.

Jenkins, P.V., Pasi, K.J. & Perkins, S.J. (1998) Molecular modeling of ligand and mutation sites of the type A domains of human von Willebrand factor and their relevance to von Willebrand's disease. *Blood*, **91**, 2032–2044.

Kokame, K., Matsumoto, M., Soejima, K., Yagi, H., Ishizashi, H., Funato, M., Tamai, H., Konno, M., Kamide, K., Kawano, Y., Miyata, T. & Fujimura, Y. (2002) Mutations and common polymorphisms in *ADAMTS13* gene responsible for von Willebrand factor-cleaving protease activity. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 11902–11907.

Kokame, K., Matsumoto, M., Fujimura, Y. & Miyata, T. (2004) VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. *Blood*, **103**, 607–612.

Levy, G.G., Nichols, W.C., Lian, E.C., Foroud, T., McClintick, J.N., McGee, B.M., Yang, A.Y., Siemieniak, D.R., Stark, K.R., Gruppo, R., Sarode, R., Shurin, S.B., Chandrasekaran, V., Stabler, S.P., Sabio, H., Bouhassira, E.E., Upshaw, J.D. Jr, Ginsburg, D. & Tsai, H.M. (2001) Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*, **413**, 488–494.

Mannami, T., Konishi, M., Baba, S., Nishi, N. & Terao, A. (1997) Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: the Suita study. *Stroke*, **28**, 518–525.

Mannucci, P.M., Canciani, M.T., Forza, I., Lussana, F., Lattuada, A. & Rossi, F. (2001) Changes in health and disease of the metalloprotease that cleaves von Willebrand factor. *Blood*, **98**, 2730–2735.

Matsumoto, M., Kokame, K., Soejima, K., Miura, M., Hayashi, S., Fujii, Y., Iwai, A., Ito, F., Tsuji, Y., Takeda-Shitaka, M., Iwadate, M., Umeyama, H., Yagi, H., Ishizashi, H., Banno, F., Nakagaki, T., Miyata, T. & Fujimura, Y. (2004) Molecular characterization of *ADAMTS13* gene mutations in Japanese patients with Upshaw-Schulman syndrome. *Blood*, **103**, 1305–1310.

Moake, J.L., Rudy, C.K., Troll, J.H., Weinstein, M.J., Colannino, N.M., Azocar, J., Seder, R.H., Hong, S.L. & Deykin, D. (1982) Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *New England Journal of Medicine*, **307**, 1432–1435.

Moschcowitz, E. (1924) Hyaline thrombosis of the terminal arterioles and capillaries; a hitherto undescribed disease. *Proceedings of the New York Pathological Society*, **24**, 21–24.

Nishio, K., Anderson, P.J., Zheng, X.L. & Sadler, J.E. (2004) Binding of platelet glycoprotein Ibα to von Willebrand factor domain A1 stimulates the cleavage of the adjacent domain A2 by ADAMTS13. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 10578–10583.

Obert, B., Tout, H., Veyradier, A., Fressinaud, E., Meyer, D. & Girma, J.P. (1999) Estimation of the von Willebrand factor-cleaving protease in plasma using monoclonal antibodies to VWF. *Thrombosis and Haemostasis*, **82**, 1382–1385.

Pimanda, J.E., Maekawa, A., Wind, T., Paxton, J., Chesterman, C.N. & Hogg, P.J. (2004) Congenital thrombotic thrombocytopenic purpura in association with a mutation in the second CUB domain of ADAMTS13. *Blood*, **103**, 627–629.

Plaimauer, B., Zimmermann, K., Volkel, D., Antoine, G., Kerschbaumer, R., Jenab, P., Furlan, M., Gerritsen, H., Lämmle, B., Schwarz, H.P. & Scheiflinger, F. (2002) Cloning, expression and functional characterization of the von Willebrand factor-cleaving protease (ADAMTS13). *Blood*, **100**, 3626–3632.

Savasan, S., Lee, S.K., Ginsburg, D. & Tsai, H.M. (2003) *ADAMTS13* gene mutation in congenital thrombotic thrombocytopenic purpura with previously reported normal VWF cleaving protease activity. *Blood*, **101**, 4449–4451.

Schneppenheim, R., Budde, U., Oyen, F., Angerhaus, D., Aumann, V., Drewke, E., Hassenpflug, W., Haberle, J., Kentouche, K., Kohne, E., Kurnik, K., Mueller-Wiefel, D., Obser, T., Santer, R. & Sykora, K.W. (2003) von Willebrand factor cleaving protease and *ADAMTS13* mutations in childhood TTP. *Blood*, **101**, 1845–1850.

Soejima, K., Mimura, N., Hirashima, M., Maeda, H., Hamamoto, T., Nakagaki, T. & Nozaki, C. (2001) A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? *Journal of Biochemistry*, **130**, 475–480.

Studt, J.D., Böhm, M., Budde, U., Girma, J.P., Varadi, K. & Lämmle, B. (2003) Measurement of von Willebrand factor-cleaving protease (ADAMTS-13) activity in plasma: a multicenter comparison of different assay methods. *Journal of Thrombosis and Haemostasis*, **1**, 1882–1887.

Sutherland, J.J., O'Brien, L.A., Lillicrap, D. & Weaver, D.F. (2004) Molecular modeling of the von Willebrand factor A2 domain and the effects of associated type 2A von Willebrand disease mutations. *Journal of Molecular Modeling (Online)*, 10, 259–270.

Tripodi, A., Chantarangkul, V., Böhm, M., Budde, U., Dong, J.F., Friedman, K.D., Galbusera, M., Girma, J.P., Moake, J., Rick, M.E., Studt, J.D., Tureček, P.L. & Mannucci, P.M. (2004) Measurement of von Willebrand factor cleaving protease (ADAMTS-13): results of an international collaborative study involving 11 methods testing the same set of coded plasmas. *Journal of Thrombosis and Haemostasis*, 2, 1601–1609.

Tsai, H.M. (1996) Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood*, **87**, 4235–4244.

Tsai, H.M. & Lian, E.C. (1998) Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *New England Journal of Medicine*, **339**, 1585–1594.

Tsai, H.M., Sussman, I.I. & Nagel, R.L. (1994) Shear stress enhances the proteolysis of von Willebrand factor in normal plasma. *Blood*, **83**, 2171–2179.

Vesely, S.K., George, J.N., Lämmle, B., Studt, J.D., Alberio, L., El-Harake, M.A. & Raskob, G.E. (2003) ADAMTS13 activity in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: relation to presenting features and clinical outcomes in a prospective cohort of 142 patients. *Blood*, **102**, 60–68.

Veyradier, A., Obert, B., Houllier, A., Meyer, D. & Girma, J.P. (2001) Specific von Willebrand factor-cleaving protease in thrombotic microangiopathies: a study of 111 cases. *Blood*, **98**, 1765–1772.

Zheng, X., Chung, D., Takayama, T.K., Majerus, E.M., Sadler, J.E. & Fujikawa, K. (2001) Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *Journal of Biological Chemistry*, **276**, 41059–41063.

Zheng, X.L., Kaufman, R.M., Goodnough, L.T. & Sadler, J.E. (2004) Effect of plasma exchange on plasma ADAMTS13 metalloprotease activity, inhibitor level, and clinical outcome in patients with idiopathic and nonidiopathic thrombotic thrombocytopenic purpura. *Blood*, **103**, 4043–4049.

Characterization of a core binding site for ADAMTS-13 in the A2 domain of von Willebrand factor

EXHIBIT 3

Jing-Jiang Wu, Kazuo Fujikawa, Brad A. McMullen, and Dominic W. Chung*

Department of Biochemistry, University of Washington, Seattle, WA 98195

Communicated by Bert L. Vallee, Harvard Medical School, Boston, MA, October 18, 2006 (received for review September 29, 2006)

ADAMTS-13, a metalloprotease in plasma, specifically cleaves the Tyr-1605–Met-1606 bond in the A2 domain of von Willebrand factor (VWF) to regulate the polymer distribution of VWF in circulation, which is critical for primary hemostasis. A 73-aa peptide (VWF73) was previously identified as the minimal substrate cleavable by ADAMTS-13. In this study, VWF73 was enzymatically and chemically cleaved into shorter peptides, and the inhibition of cleavage of a VWF73-derived substrate by these purified peptides was measured in competition studies using a quantitative assay we recently reported. A 24-aa peptide encompassing Pro-1645–Lys-1668 (P'40–P'63) and situated 40 aa downstream from the cleavage site was the minimal peptide that could bind to and competitively inhibit ADAMTS-13 (K_i = 12 μM). This peptide and longer peptides encompassing this core sequence also inhibited the cleavage of multimeric VWF by ADAMTS-13. These results suggest the presence of a complementary extended binding site, or exosite, on ADAMTS-13. Mutation of Asp-1653 and Asp-1663 to Ala in this region significantly reduced the rate of cleavage of the substrate peptide, whereas the Glu1655Ala mutation caused an enhanced rate of cleavage. These results suggest that ionic interactions of the Pro-1645–Lys-1668 region with the exosite on ADAMTS-13 play a significant role in mediating substrate recognition.

ADAMTS-13 inhibition | exosite

The polymeric adhesive protein von Willebrand factor (VWF) plays a critical role in primary hemostasis (1). Its hemostatic function depends on the unique composition of multimers: large multimeric forms are crucial for shear-dependent platelet aggregation, and small multimers, such as dimers and tetramers, are unable to support platelet aggregation. The multimer distribution of VWF in plasma is regulated in part by ADAMTS-13, a metalloprotease (2–4) that cleaves the ultra-large form of VWF (ULVWF) (5) secreted by activated endothelial cells and platelets into the less adhesive form consisting of small multimers (6). A deficiency in ADAMTS-13 leads to inadequate processing and accumulation of the highly adhesive ULVWF in circulation (7, 8). ULVWF multimers unfold under high shear in arterioles and capillaries and bind to platelets to form pathological microvascular thrombi. Deposition of these pathological thrombi in the microvasculature is a characteristic feature of the disease thrombotic thrombocytopenic purpura (TTP). Familial TTP is caused by inherited mutations in the *ADAMTS13* gene (9). A majority of patients with acute idiopathic TTP was found to contain circulating inhibitory autoantibodies to ADAMTS-13 (7, 8). Assessment of the ADAMTS-13 level in plasma and the level of inhibitory antibodies has since become an important tool in the diagnosis of TTP and in monitoring the disease process.

The ADAMTS-13 level in plasma was initially measured by directly assessing the loss of large VWF multimers (3) or the increase in abundance of cleaved fragments (4). Other indirect measurements, such as residual collagen binding (10) and residual ristocetin cofactor activity (11), also have been developed. These assays, involving lengthy procedures and the use of nonphysiological denaturants, are difficult to perform and often have large interassay variations. Kokame *et al.* (12) showed that a 73-aa peptide from the A2 domain of VWF (VWF73) was cleaved efficiently by ADAMTS-13. Cleavage of this peptide was more efficient than the entire A2 domain of VWF. It was hypothesized that VWF73 assumes a conformation comparable with that of the shear-induced accessible conformation of the A2 domain in VWF multimers. It was unusual that an extended peptide sequence, consisting of 10 aa before and 63 aa after the scissile bond, is necessary for recognition and cleavage by ADAMTS-13. To understand how ADAMTS-13 interacts with this extended peptide substrate, we further fragmented VWF73 into shorter peptides and identified regions that contribute to substrate recognition.

Results

A derivative of VWF73, consisting of a HRP conjugate of a biotinylated VWF78 sequence, previously designated as HRP-H-A2-B (13), was used as substrate for characterizing the activity of ADAMTS-13 in the presence of peptides derived from various regions of the A2 domain of VWF. In the absence of inhibitory peptides, HRP-H-A2-B was cleaved efficiently, with a K_m of 0.25 $\pm$ 0.03 μM, K_{cat} of 0.67 s^{-1}, and a catalytic efficiency (k_{cat}/K_m) of 3.05 $\times$ 10^6 $M^{-1}s^{-1}$ as determined by nonlinear least square curve fitting. The recombinant A2 domain of VWF (Val-1476–Gly-1672) was digested with cyanogen bromide to generate a mixture of peptides. This mixture exhibited strong inhibitory activity on the cleavage of HRP-H-A2-B by ADAMTS-13 (data not shown), indicating that treatment with cyanogen bromide did not destroy sequences that could bind to ADAMTS-13. Cyanogen bromide-derived peptides of A2 were separated by HPLC, and the separated peptides were individually tested for inhibitory activity. Only one tryptophan-containing peptide, encompassing Val-1607–Gly-1672, inhibited cleavage of HRP-H-A2-B (data not shown). Because this peptide is contained in VWF73, we carried out subsequent quantitative studies with peptides derived from H-A2-B.

H-A2-B was digested with cyanogen bromide, endopeptidase Lys-C, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine (BNPS-skatole), and trypsin, and the resulting peptides were purified by HPLC (Fig. 1). The purity and identity of the peptides were confirmed by mass spectrometry. Cleavage of H-A2-B after the single Met-1606 by cyanogen bromide generated two peptides, M3 and M6. M6, which is 1 aa shorter than

Author contributions: J.-J.W., K.F., and D.W.C. designed research; J.-J.W., K.F., and B.A.M. performed research; J.-J.W., K.F. and B.A.M. contributed new reagents/analytic tools; J.-J.W., K.F., B.A.M., and D.W.C. analyzed data; and D.W.C. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Abbreviations: VWF, von Willebrand factor; TTP, thrombotic thrombocytopenic purpura; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine.

*To whom correspondence should be addressed. E-mail: chung@u.washington.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0609150103

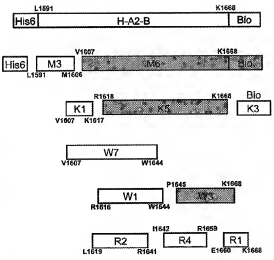


Fig. 1. Schematic representation of peptides derived from H-A2-B. Peptides are represented by the bars. Amino acids represented by their single-letter codes at the beginning and end of each peptide were numbered according to the sequence in VWF. Peptides with inhibitory activities are shown in gray.

the product derived from cleavage of H-A2-B at the Tyr-1605–Met-1606 bond by ADAMTS-13, competitively inhibited the cleavage of HRP-H-A2-B (Fig. 2), with an inhibition constant (K_i) of 0.25 ± 0.04 μM as determined by nonlinear least-square curve fitting (Table 1). M3, the sequence from the P10–P′1 positions, did not show any inhibitory activity at concentrations up to 45 μM.

M6 was further cleaved by endopeptidase Lys-C into peptides K1, K3, and K5, and only K5 competitively inhibited ADAMTS-13 (K_i = 1.18 ± 0.52 μM) (Table 1). Because K3, the C-terminal biotinylated tag sequence, did not show any inhibition, this region was not responsible for the inhibitory activity observed with M6. Equimolar mixtures of K1 and K5 did not restore the low K_i of M6 (Fig. 3*A*), indicating that K1 must be covalently linked to K5 to show high affinity for ADAMTS-13. These results showed that the removal of K1 sequences in the P′2–P′12 positions is associated with a 5-fold decrease in affinity.

K5 was further cleaved at Trp-1644 by BNPS-skatole, and the resulting peptides W1 and W3 were purified and studied. Only W3 competitively inhibited ADAMTS-13 (K_i = 12 ± 2.5 μM) (Table 1). Neither W1 nor W7, derived from BNPS-skatole cleavage of M6, inhibited ADAMTS-13. In mixing studies, the extent of inhibition by an equimolar mixture of W1 and W3 (Fig. 3*B*) did not differ significantly from that of W3 alone, indicating that W1 can only enhance the binding of W3 to ADAMTS-13 when it was covalently linked to W3. Removal of W1, corresponding to positions P′13–P′39, resulted in another 10-fold decrease in affinity for ADAMTS-13.

K5 was cleaved by trypsin into R1, R2, and R4, and none of these tryptic peptides exhibited appreciable inhibition of ADAMTS-13 (Table 1). An equimolar mixture of R1 and R4 also failed to reconstitute competitive inhibition comparable with that by W3. These studies showed that the smallest peptide identified in this study that could independently bind to ADAMTS-13 was W3, which is situated at positions P′40–P′63 from the cleavage site. The presence of sequences from P′2–P′39 positions increased the affinity for ADAMTS-13. However, sequences from this region by themselves, such as the isolated peptides W7, K1, and W1, were unable to bind to ADAMTS-13 with high affinity unless they were linked to W3.

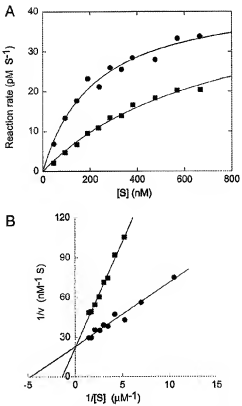


Fig. 2. Inhibition of HRP-H-A2-B cleavage by M6. (*A*) Rate of substrate cleavage in the absence (●) and presence (■) of 0.6 μM M6. (*B*) Lineweaver–Burk plot of data shown in *A*. ●, absence of M6; ■, presence of M6.

The three inhibitory peptides, M6, K5, and W3, also inhibited the cleavage of multimeric VWF by ADAMTS-13 (Fig. 4). Increasing concentrations of these peptides progressively inhibited the conversion of large VWF multimers to dimers and trimers, consistent with the notion that the binding site on ADAMTS-13 for these inhibitory peptides is involved in the recognition of the multimeric VWF substrate. The concentration of W3 that showed significant inhibition was higher than those

Table 1. Inhibition constants of peptides from Leu-1591 to Lys-1668 of VWF

Peptide	Position	K_i, μM
M3	P15–P′1	(>45)
M6	P′2–P′63	0.25 ± 0.04
K1	P′2–P′12	(>46)
K5	P′13–P′63	1.18 ± 0.52
K3	Biotin acceptor	—
W1	P′13–P′39	(>238)
W3	P′40–P′63	12 ± 2.5
W7	P′2–P′39	(>31)
R2	P′13–P′35	(>78)
R4	P′36–P′53	(>78)
R13	P′54–P′63	(>78)

The peptides shown correspond to those in Fig. 1, and the peptide positions are relative to the scissile P1–P′1 Tyr-1605–Met-1606 bond. Concentrations in parentheses represent the highest concentrations tested that showed no inhibition.

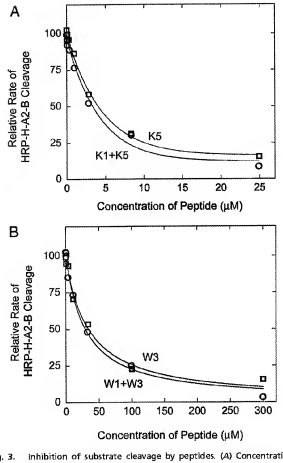


Fig. 3. Inhibition of substrate cleavage by peptides. (*A*) Concentration-dependent inhibition of K5 (□) and equimolar mixtures of K1 and K5 (○) at a substrate concentration of 0.07 μM. (*B*) Concentration-dependent inhibition of W3 (□) and equimolar mixtures of W1 and W3 (○) at a substrate concentration of 0.07 μM.

of M6 and K5, consistent with the higher K_i for this peptide determined in the inhibition studies.

Cleavage of multimeric VWF and VWF73 by ADAMTS-13 was sensitive to high ionic strength, particularly chloride anions (14). We reasoned that ionic interactions may play a role in the interaction of ADAMTS-13 with its substrate, including the core sequence W3. The contribution of the six charged amino acids in the W3 region, Asp-1653, Glu-1655, Arg-1659, Glu-1660, Asp-1663, and Lys-1668, was assessed individually in H-A2-B by alanine mutation analysis. In these studies, the rate of cleavage by partially purified ADAMTS-13 was quantitated by SDS/PAGE, followed by staining with Sypro orange, and fluorescence intensity quantitation by fluorescence imaging. Although Arg1659Ala, Glu1660Ala, and Lys1668Ala mutations in H-A2-B did not significantly change the cleavage rate by ADAMTS-13, Asp1653Ala and Asp1663Ala mutations significantly reduced the rate of cleavage by 60% and 42%, respectively (Fig. 5). Interestingly, the Glu1655Ala mutation enhanced the rate of cleavage by 24%. These results are in agreement with the idea that ionic interactions involving residues 1653, 1655, and 1663 play a role in substrate recognition in the P′40–P′63 region.

Discussion

Studies on the initial rates of cleavage of HRP-H-A2-B showed that the reaction followed typical Michaelis–Menten kinetics

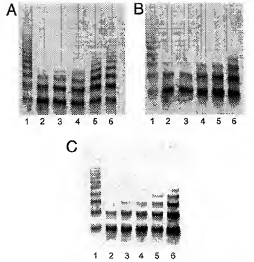


Fig. 4. Cleavage of multimeric VWF by ADAMTS-13 in the presence of inhibitory peptides. (*A*) Inhibition by M6. Lane 1, VWF without digestion; lane 2, VWF digested with ADAMTS-13 in plasma; lane 3, digestion with 2.1 μM M6; lane 4, 4.2 μM M6; lane 5, 8.4 μM M6; lane 6, 12.6 μM M6. (*B*) Inhibition by K5. Lanes 1 and 2, same as in *A*; lane 3, 2.1 μM K5; lane 4, 4.2 μM K5; lane 5, 11 μM K5; lane 6, 21 μM K5. (*C*) Inhibition by W3. Lanes 1 and 2, same as in *A*; lane 3, 11 μM W3; lane 4, 22 μM W3; lane 5, 43 μM W3; lane 6, 65 μM W3.

with a K_m of 0.25 μM. This K_m value is approximately 6-fold lower than that of VWF76 (K_m = 1.6 μM) (15), and 13-fold lower than that of the fluorescent substrate FRETS-VWF73 (K_m = 3.2 μM) (16). The difference with the VWF76 substrate may be attributed to the dissimilarity in assay conditions. In this regard, although measurements in this study were carried out in reactions containing 5 mM Hepes, studies on VWF76 were carried out in the presence of 150 mM NaCl, which was known to inhibit the activity of ADAMTS-13 *in vitro* (14). The difference with the FRETS-VWF73 substrate may be due to the substitution of amino acids in the P7 and P′5 positions for the introduction of a fluorescence-emitting group and a corresponding quenching group to promote fluorescence energy transfer in FRETS-VWF73. Another possibility is that covalent attachment of HRP to H-A2-B at a unique site N-terminal to the VWF73 sequence may have oriented VWF73 in a favorable conformation for cleavage. The low K_m exhibited by the HRP-conjugated substrate

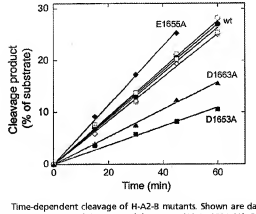


Fig. 5. Time-dependent cleavage of H-A2-B mutants. Shown are data for wild type (wt, ●), D1653A (■), D1663A (▲), E1655A (◆), R1659A (○), E1660A (□), and K1668A (○).

HRP-H-A2-B enabled us to carry out cleavage rate measurements with a smaller amount of ADAMTS-13, at room temperature, and in brief incubations.

Initial studies on peptides derived from cyanogen bromide digestion of the entire A2 domain of VWF showed that sequences outside the VWF73 region had no effect on the activity of ADAMTS-13. This is consistent with previous truncation studies (12) showing that removal of these sequences promotes exposure and cleavage of the VWF73 region. Peptides outsides the VWF73 region also did not enhance cleavage of VWF73-derived substrate, ruling out the possibility that these sequences would bind to sites in ADAMTS-13 that allosterically regulate proteolytic activity.

Peptide W7 showed no inhibition at concentrations up to 31 μM. We were unable to assess the properties of this peptide at higher concentrations due to its limited recovery from the BNPS-skatole digest of M6. It is possible that this peptide would exhibit inhibition at higher concentrations. Nevertheless, the K_i would be significantly higher than those of the three peptides M6, K5, and W3, encompassing the core binding region.

Of all of the peptides studied, M6, which is 1 aa shorter than the product of proteolysis, exhibited the strongest inhibition. The K_i of 0.25 μM for M6 indicated that it competed favorably with the substrate (K_m = 0.25 μM) under the conditions used in this study. This strong inhibition by M6 also suggests that product inhibition might be a key feature in the regulation of ADAMTS-13 activity *in vivo*. Consistent with strong product inhibition, cleavage of multimeric VWF by ADAMTS-13, promoted by the presence of denaturants, rarely approach completion despite prolonged incubation. Together with shear-induced exposure of the cleavage site, product inhibition may regulate the extent of proteolysis and the multimer distribution of VWF in circulation.

The progressive increase in K_i that correlates with deletion of sequences in the P′2–P′39 region confirms that, although sequences in this region did not bind to ADAMTS-13 by themselves, they improve affinity of the core W3 sequence in the P′40–P′63 region when they were linked. These results are consistent with the proposal that substrate interaction initiates with binding of the core sequence in the P′40–P′63 region, which aligns sequences in the contiguous P′2–P′39 region for additional interaction, and positions the scissile bond to the active center of the enzyme. Also consistent with the notion that the entire P′2–P′63 region interacts with ADAMTS-13, mutations in P′33 and P′35 led to an approximate 2-fold decrease in the catalytic efficiency, and mutations in the P′9, P′10, and P′12 positions led to a 3- to 6-fold reduction in the catalytic efficiency (15).

The requirement of an extended substrate sequence and evidence of interaction along its length with ADAMTS-13 suggests that there is a complementary extended binding site, or exosite, on ADAMTS-13 adjacent to or contiguous with the catalytic center. The location of this exosite on ADAMTS-13 is not known. It has been shown that autoimmune antibodies from acquired TTP patients interact with a common epitope(s) located in the Cys-rich and spacer domain of ADAMTS-13. Deletion of the Cys-rich and spacer domains in recombinant ADAMTS-13 also resulted in loss of proteolytic activity toward multimeric VWF and peptide substrates, such as VWF73. These observations suggest that the exosite in ADAMTS-13 that plays a role in substrate recognition may involve sequences in the Cys-rich and spacer domain of ADAMTS-13. Specific inhibition of ADAMTS-13 by targeting the catalytic center and this putative exosite may be one way of reducing the excessive degradation of VWF in patients with type 2A von Willebrand disease, in whom mutations predispose the VWF molecules to excessive degradation by ADAMTS-13 (reviewed in ref. 17).

Materials and Methods

Peptide Fragmentation. Peptides (1–5 mg) were cleaved with 2% cyanogen bromide in 10% formic acid and 6 M guanidine hydrochloride for 18 h as described (18). Digestion of peptides with endoprotease Lys-C and trypsin (Roche Diagnostics, Indianapolis, IN) was performed in the presence of 0.1 M ammonium bicarbonate with enzyme to peptide weight ratios of 200:1 and 100:1, respectively (19). Cleavage of peptides with BNPS-skatole was performed as described (20).

Peptide Isolation and Quantitation. Peptides were purified by reverse-phase HPLC on a C-18 column (μBondapak; Waters, Milford, MA) connected to a Waters HPLC system. Peptides, eluted with a 0–80% acetonitrile gradient (21), were detected by absorbance at 214 or 280 nm. The peptides (1 μg) were analyzed by liquid chromatography-electrospray ionization mass spectrometry at the Mass Spectrometry Center of the University of Washington. The concentrations of peptides were determined by amino acid analysis using fluorescamine (22) on the hydrolysates obtained after incubation of the peptides in 6 M HCl under argon at 110°C for 20 h. The concentrations of large peptides, H-A2-B, M6, and K5, were also determined by the BCA agent (Pierce, Rockford, IL). Trp-containing peptides were also quantitated spectrophotometrically by using an ε_{280} of 5.56.

Inhibition Studies. Kinetic analyses on reaction rates in the presence of peptides were performed under two conditions. Initially, various amounts of peptide were added to a fixed amount of substrate for the determination of IC_{50}. Reaction rates were determined as described (13). Subsequently, with peptides that showed inhibition, additional studies were performed by varying the substrate concentration in the presence of a fixed inhibitory peptide concentration. The reaction rates were fitted to the appropriate equations by nonlinear least squares with KaleidaGraph. Kinetic data were also analyzed in Lineweaver–Burke plots to verify competitive inhibition.

Mutagenesis. Site-directed mutagenesis of H-A2-B was performed by PCR with a QuikChange II mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide primers used for mutagenesis were 5′-TATCCTCATCCAGGCTTTGAGACGCTCGC-3′ for the Asp1653Ala mutation, 5′-ATCCAGGACTTTGCGACGCTC-CCCCGA-3′ for the Glu1655Ala mutation, 5′-TTTGAGACGCT-CCCGCAGAGGCTCCTGA-CCT-3′ for the Arg1659Ala mutation, 5′-ACGCTCCCCCG-AGCGGCTCCTGACCT-3′ for the Glu1660Ala mutation, 5′-GAGAGGCTCCTGCCCTGGTGCT-GCAG-3′ for the Asp1663Ala mutation, and 5′-GACCTGGT-GCTGCAGGCGCTTCTGAACGACAT-3′ for the Arg1668Ala mutation. All mutations were confirmed by DNA sequencing. Mutant H-A2-B peptides were expressed and purified as described (13).

Digestion of Multimeric VWF. Normal human plasma was desalted on a PD-10 column (GE Healthcare, Piscataway, NJ) in 10 mM Hepes, pH 7.4. VWF was digested with the ADAMTS-13 in the desalted plasma by incubation for 15 h at 37°C in 5 mM Hepes, pH 7.4/7 mM $CaCl_2$/1 M urea with or without peptides. The reaction was terminated by methanol (final concentration of 10%) and placed on ice for 30 min. Precipitates were collected by centrifugation at 8,000 × *g* for 10 min and dissolved in gel sample buffer (50 mM Tris·HCl, pH 6.8/8 M urea/2 mM EDTA). The samples were heated at 80°C for 10 min and applied to 1% agarose gels prepared according to the method of Warren *et al.* (23). The separated VWF multimers were transferred to a PVDF membrane and visualized by the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Kirkegaard & Perry Laboratorics, Gaithersburg, MD) after

incubation with a polyclonal rabbit anti-human VWF antibody (Accurate Chemical and Scientific, Westbury, NY) and alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO).

Digestion of Mutant Substrate Peptides. Normal and mutant H-A2-B substrate peptides were reduced by DTT and reacted with iodoacetamide as described (24). The blocked peptides were purified by HPLC as described above. Peptides (1.5 μM) were digested with partially purified ADAMTS-13 (equivalent to 0.1 μg/ml) (25) at 22°C, and aliquots were withdrawn at 15-min intervals. The digestion was stopped by the addition of SDS/PAGE sample buffer containing EDTA and DTT (final concentrations of 10 and 20 mM, respectively). The digested products were separated by SDS/PAGE using 16% polyacrylamide gels containing tricine (Invitrogen, Carlsbad, CA). The separated peptides were stained with the fluorescent dye Sypro orange diluted in 10% acetic acid as recommended by the manufacturer (Invitrogen), and the intensity of blue fluorescence (excitation at 450 nm) was determined in a Storm 840 imaging system (GE Healthcare) and analyzed with the ImageQuant 5.2 program.

We thank Dr. Earl Davie for advice and suggestions and Jeff Harris for technical assistance. This work was supported by National Institutes of Health Grant HL070681.

1. Sadler JE (1998) *Annu Rev Biochem* 67:395–424.
2. Dent JA, Berkowitz SD, Ware J, Kasper CK, Ruggeri ZM (1990) *Proc Natl Acad Sci USA* 87:6306–6310.
3. Furlan M, Robles R, Lammle B (1996) *Blood* 87:4223–4234.
4. Tsai HM (1996) *Blood* 87:4235–4244.
5. Moake JL (2004) *Semin Hematol* 41:4–14.
6. Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, Schade AJ, McIntire LV, Fujikawa K, Lopez JA (2002) *Blood* 100:4033–4039.
7. Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, Krause M, Scharrer I, Aumann V, Mittler U, *et al.* (1998) *N Engl J Med* 339:1578–1584.
8. Tsai HM, Lian EC (1998) *N Engl J Med* 339:1585–1594.
9. Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, Yang AY, Siemieniak DR, Stark KR, Gruppo R, *et al.* (2001) *Nature* 413:488–494.
10. Gerritsen HE, Turecek PL, Schwarz HP, Lammle B, Furlan M (1999) *Thromb Haemost* 82:1386–1389.
11. Bohm M, Vigh T, Scharrer I (2002) *Ann Hematol* 81:430–435.
12. Kokame K, Matsumoto M, Fujimura Y, Miyata T (2004) *Blood* 103:607–612.
13. Wu JJ, Fujikawa K, Lian EC, McMullen BA, Kulman JD, Chung DW (2006) *J Thromb Haemost* 4:129–136.
14. De Criscofaro R, Peyvandi F, Palla R, Lavoretano S, Lombardi R, Merati G, Romitelli F, Di Stasio E, Mannucci PM (2005) *J Biol Chem* 280:23295–23302.
15. Zanardelli S, Crawley JT, Chion CK, Lam JK, Preston RJ, Lane DA (2006) *J Biol Chem* 281:1555–1563.
16. Anderson PJ, Kokame K, Sadler JE (2006) *J Biol Chem* 281:850–857.
17. Sadler JE (2005) *J Thromb Haemost* 3:1702–1709.
18. McMullen BA, Fujikawa K, Davie EW (1991) *Biochemistry* 30:2050–2056.
19. McMullen BA, Fujikawa K (1985) *J Biol Chem* 260:5328–5341.
20. Ornstein GS, Fontana A, Anfinsen CB (1970) *J Biol Chem* 245:1895–1902.
21. McMullen BA, Fujikawa K, Davie EW, Hedner U, Ezban M (1995) *Protein Sci* 4:740–746.
22. Nakai N, Lai CY, Horecker BL (1974) *Anal Biochem* 58:563–570.
23. Warren CM, Krzesinski PR, Greaser ML (2003) *Electrophoresis* 24:1695–1702.
24. Crestfield AM, Moore S, Stein WH (1963) *J Biol Chem* 238:622–627.
25. Fujikawa K, Suzuki H, McMullen B, Chung D (2001) *Blood* 98:1662–1666.

EXHIBIT 4

ADAMTS13

Name	Description	REF

ACTIFLUOR™ ADAMTS13 Activity — 012

A FRET-based assay for the measurement of ADAMTS13 activity in human plasma. ADAMTS13 is a metalloproteinase that cleaves ultra large (UL) vWF multimers within the A2 region of vWF. A deficiency or low level of ADAMTS13 activity (<5%) may lead to an accumulation of UL-vWF multimers. Low levels of ADAMTS13 activity are associated with Thrombotic Thrombocytopenia Purpura (TTP).

Specifications:		**Kit Components:**
Samples:	Only citrate collected plasma	48 white fluorescence microtiter wells
	Do not use EDTA collected plasma.	FRET substrate
Sample Size:	5 - 10 µL	Assay buffer
Assay Time:	30 - 60 minutes	Standard
No. of Tests:	48	Positive control

IMUBIND® ADAMTS13/fXI Complex ELISA — 011

Measures ADAMTS13/fXI complexes in human plasma. ADAMTS13 is a metalloproteinase that cleaves ultra large (UL) vWF multimers within the A2 region of vWF. A deficiency or low level of ADAMTS13 activity (<5%) may lead to an accumulation of UL-vWF multimers. Low levels of ADAMTS13 activity are associated with Thrombotic Thrombocytopenia Purpura (TTP). Studies have identified patients with clinical symptoms of TTP that possess normal levels of ADAMTS13 and patients that possess low ADAMTS13 activity without symptoms of TTP. ADAMTS13 forms a stable complex with Factor XI (fXI) and Factor XIa (fXIa), and measurements of these complexes in plasma may be useful toward the understanding of the role ADAMTS13 and fXI play in the etiology and biology of congenital and/or acquired TTP

Specifications:		**Kit Components:**
Samples:	Only citrate collected plasma	96-well antibody coated plate
	Do not use EDTA collected plasma.	Assay buffer
Sample Preparation:	1:10 dilution	Plasma standards
Sample Size:	100 µL	Positive control
Assay Time:	4 hours	Detection antibody
Standard Range:	0.78% - 25% (normal plasma is 100%)	Substrate
Sensitivity:	ND	Wash buffer
Precision:	Intra-assay CV = 5.6%	

IMUBIND® ADAMTS13 ELISA — CE Pending — 013

Measures ADAMTS13 protein in human plasma. ADAMTS13 is a metalloproteinase that cleaves ultra large (UL) vWF multimers within the A2 region of vWF. A deficiency or low level of ADAMTS13 activity (<5%) may lead to an accumulation of UL-vWF multimers. Studies have shown that low levels of ADAMTS13 activity are associated with Thrombotic Thrombocytopenia Purpura (TTP), a life-threatening hematological condition characterized by low platelet count, microvascular thrombi, red cell fragmentation, CNS and renal complications.

Specifications:		**Kit Components:**
Samples:	Only citrate collected plasma	96-well antibody coated plate
	Do not use EDTA collected plasma.	Assay buffer
Sample Preparation:	1:10 dilution	Plasma standards
Sample Size:	100 µL	Positive control
Assay Time:	5 hours	Detection antibody
Standard Range:	0 - 200 ng/mL	Enzyme conjugate
Sensitivity:	ND	Substrate
Precision:	Intra-assay CV = 4.0%	Wash buffer
	Inter-assay CV = 7.3%	

ADAMTS-13 Activity Assay (ATS-13®)

INTENDED USE

For the quantitative measurement of ADAMTS-13 protease activity.

For Research use Only.

SUMMARY AND EXPLANATION

It was recently discovered that ADAMTS-13 is the protease responsible for cleaving von Willebrand Factor; deficiency of ADAMTS-13 activity has been demonstrated in the plasma of thrombotic thrombocytopenic purpura (TTP) patients. The lack of ADAMTS-13 activity results in the accumulation of multimers of von Willebrand Factor in the plasma and ultimately intravascular platelet aggregation resulting in the clinical symptoms associated with TTP.[4,5] Mild or moderately decreased levels of ADAMTS-13 activity have also been associated with other disease states and conditions.[2-5]

PRINCIPLE OF THE PROCEDURE

The ATS-13® assay is based on fluorescence resonance energy transfer (FRET) technology. A synthetic fragment of the von Willebrand Factor protein is used as the Substrate. Cleavage of this peptide between two modified residues releases the fluorescence quenching capabilities.

This assay is based on quantifying the cleavage of a small fragment of von Willebrand Factor by the ADAMTS-13 protease. The cleavage of this synthetic substrate is detected by reading the fluorescence that results when the substrate is cleaved.

REAGENTS

Maximum number of tests per kit: 40
All reagents should be stored as directed by the label.

ATS-MS	1. Black Microwell Strips: Once removed from the foil pouch, take care not to expose the strips to dust or particulates. Take care to protect from moisture. Strips should be stored at room temperature. Ready to use.
ATS-SUB	2. Substrate: lyophilized . Keep substrate protected from light. Store lyophilized material at -15 to -30°C. Hydrated substrate should be stored upright in the parafilmed original stoppered vial at -15 to -30°C (non-cycling freezer) in the dark.
ATS-SD	3. Specimen Diluent: Ready for use. Store at 2 to 8°C.
ATS-SB	4. Substrate Buffer: Ready for use. Store at 2 to 8°C.
ATS-PCH	5. Positive Control; HIGH: Store at -15 to -30 °C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13® Calibrator and Control Recording Sheet. Discard after single use.
ATS-PCL	6. Positive Control; LOW: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13 ® Calibrator and Control Recording Sheet. Discard after single use.
ATS-CALA	7. Calibrator A: Store at -15 to -30°C. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13 ® Calibrator and Control Recording Sheet. Discard after single use.
ATS-CALB	8. Calibrator B: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13 ® Calibrator and Control Recording Sheet. Discard after single use.

ATS-CALC	9.	Calibrator C: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13 ® Calibrator and Control Recording Sheet. Discard after single use.
ATS-CALD	10.	Calibrator D: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13 ® Calibrator and Control Recording Sheet. Discard after single use.
ATS-CALE	11.	Calibrator E: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13 ® Calibrator and Control Recording Sheet. Discard after single use.

PRECAUTIONS

- Do not use reagents that are turbid or contaminated.
- Care MUST be taken to avoid contamination of Calibrators and Substrate. Inadvertent contamination of these reagents with human plasma will invalidate the assigned values of the calibrators.
- Unopened and lyophilized reagents are stable until the expiration date printed on the box when stored as directed.
- Do not use reagents beyond their expiration date.
- Microwells and reagents contained in the kit are not to be used in conjunction with any other test system.
- Discard any unused portions of Calibrators, Controls, and used Black Microwell Strips after each run.
- Substitution of components other than those provided in this kit may lead to inconsistent or erroneous results.
- When making dilutions, follow pipette manufacturer's instructions for appropriate dispensing and rinsing techniques.
- The enzyme substrate reaction is temperature sensitive and should be performed in a controlled area at 22 to 25°C.
- Only plasma should be used in the assay. Serum will give inaccurate results.

CAUTIONS

- All human plasma used in the Calibrators and Positive Controls for this product has been tested and found negative for antibody to HIV, HCV and HBsAg by FDA approved methods. No test method, however, can offer complete assurance that HIV, Hepatitis C virus, Hepatitis B virus or other infectious agents are absent. Therefore, these materials should be handled as potentially infectious.
- Discard all components when completed according to local regulations.

SPECIMEN COLLECTION

Sample Collection and Preparation

NOTE: Only platelet poor plasma collected in 3.2% sodium citrate may be used for this assay. Do not use plasma that has been collected in or treated with EDTA. See Collection, Transport and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays. Approved Guideline H21-A4 NCCLS, Volume 23, Number 35, December 2003 for details.

Plasma collection should be performed as follows:

1. Collect blood in buffered sodium citrate (light blue top, 3.2%) plastic tubes (available in 4.5 mL, 2.7 mL or 1.8 mL full draw tubes).

NOTE: Partial draw tubes should NOT be processed. Since the tubes are pre-calibrated to draw the specified amount of blood, the resulting sample, will not have the proper 9:1 ratio of blood to anticoagulant if a full sample is not collected.

2. After collection, store tube upright at room temperature until centrifugation.

NOTE: Blood samples should be centrifuged between fifteen minutes and two hours after blood collection for best results.

3. Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times.

4. Centrifuge blood sample at room temperature in a horizontal rotor (swing-out rotor) for 15 - 20 minutes at 1500 to 1800 RCF (Relative Centrifugal Force) with the brake off.

WARNING: Excessive centrifuge speed (over 2000 RCF) may cause tube breakage and exposure to blood and possible injury.

5. Following centrifugation, transfer the top 2/3 of the plasma layer into a new plastic tube.

6. Re-centrifuge the collected plasma at 1500 to 1800 RCF with the brake off for an additional 15 - 20 minutes to remove any red cells or platelets.

7. Transfer the top 2/3 of the plasma into a new plastic tube, taking care not to disturb any cells at the bottom of the tube.

Sample Storage

1. Plasma should be stored at 2 to 8°C and assayed within 4 hours OR aliquoted and frozen at -70°C or colder for up to 6 months.

2. Frozen plasma should be thawed rapidly at 37°C. Thawed plasma should be stored at 2 to 8°C and assayed within 4 hours.

PROCEDURE

Materials Provided:

Box A

1. 6 x 100 µL Positive Control: High
2. 6 x 100 µL Positive Control: Low
3. 6 sets of Calibrators, 5 levels, 100 µL each: Calibrator A, Calibrator B, Calibrator C, Calibrator D, Calibrator E.
4. 1 x 0.12 mg Substrate

Box B

1. 2 Microwell frames, each containing 6 – 2 x 8 Black Microwell Strips
2. 1 x 14 mL Specimen Diluent
3. 1 x 14 mL Substrate Buffer

Additional Materials Required:

1. Polypropylene plastic test tubes for patient sample dilutions and substrate dilution
2. Transfer pipets
3. Adjustable micropipets to deliver 10 - 100 µL and 100 – 1000 µL
4. Disposable tips
5. DMSO (Reagent Grade)
6. Fluorescent plate reader capable of measuring fluorescence at Excitation = 340 - 350 nm and Emission = 440 - 450 nm
7. Timer
8. Centrifuge
9. Aluminum Foil

Test Procedure

1. Allow all reagents to warm to room temperature.

NOTE: Only remove one foam strip set of Calibrators and Controls per assay.

2. Determine the number of patient samples to be tested. Using the Recording Sheet, assign each sample to a location consisting of two (duplicate) wells. Record the identity of each sample on the Recording Sheet. Place the sample replicates horizontally (e.g. CALA in wells A1 and A2).

3. Remove microwell frame from pouch. Promptly remove unneeded strips from frame and reseal in the protective pouch.

4. In a plastic test tube, dilute each patient plasma sample to be tested by adding 18 µL plasma into 132 µL Specimen Diluent.

5. Add 50 µL of each Calibrator (in duplicate) to the appropriate microwells of the black microwell strips as designated on the Recording Sheet. Do not dilute.

6. Add 50 µL of Positive Control: Low (in duplicate) to the appropriate microwells of the black microwell strips as designated on the Recording Sheet. Do not dilute.

7. Add 50 µL of Positive Control: High (in duplicate) to the appropriate microwells of the black microwell strips as designated on the Recording Sheet. Do not dilute.

8. Add 50 µL of the prediluted sample plasma solution (prepared in step 4) in duplicate to the appropriate microwells of the black microwell strips as designated on the Recording Sheet.

NOTE: If multiple patient samples are tested at the same time, only one set of calibrators and controls are required.

9. Prepare Stock Substrate Solution. Remove stopper carefully as some Substrate may cling to the plastic. Reconstitute the lyophilized Substrate by adding 37 µL of reagent grade DMSO to the Substrate vial. Mix solution and add 113 µL reagent grade H_2O. Replace the stopper and close the cap tightly. Mix well by gently swirling until all contents are dissolved.

10. Prepare the assay Substrate Solution (3%) in a plastic tube according to the table below:

Patient Samples to Test	Volume Stock Substrate Solution (µL)	Volume Substrate Buffer (µL)
1	25	795
5	37	1193
10	53	1714
* 40	150	4850

* This can be prepared by adding substrate buffer directly to stock substrate vial if being used for testing within one assay.

NOTE: A repeating pipette should not be used.

11. Mix the solution thoroughly. Protect from light. Immediately following preparation, add 50 µL of Substrate Solution into each microwell containing a patient sample, calibrator, or control. Gently tap the sides of the microwell frame to ensure even distribution of the Substrate Solution.

NOTE: Remaining Stock Substrate Solution should be stoppered and stored upright in the original vial with original stopper (sealed with parafilm) at –20°C (non-cycling freezer) in the dark. Re-hydrated stock can be used for up to 6 months following re-hydration.

12. Place plate in fluorimeter with Excitation = 340 - 350 nm and Emission = 440 - 450 nm at room temperature. Read and recor results as time zero.

NOTE: Reading must be taken within 5 minutes of addition of substrate.

13. Set timer for 25 minutes and start.

14. Remove plate from fluorimeter. Store plate at room temperature (not in plate reader) and protect from light for 25 - 35 minutes.

NOTE: Do not cover plate with paper or cardboard. Fibers in the plate can cause random fluorescence. Cover with aluminum foil.

15. Between 25 - 35 minutes, place plate in fluorimeter with Excitation = 340 - 350 nm and Emission = 440 - 450 nm at room temperature. Read and record results.

RESULTS

Subtract time zero fluorescence values from the 25 - 35 minute fluorescence values for all results. Construct a calibration curve by plotting the mean fluorescence (n=2) value for each calibration standard versus its corresponding concentration of ADAMTS-13 activity. A calibration curve should be generated each time the assay is performed.

CALCULATIONS

Determine the amount of ADAMTS-13 Activity in the plasma sample using the Microsoft®Excel spreadsheet provided on the enclosed CD. Instructions for the spreadsheet are on the first worksheet of the file. The spreadsheet assists with background corrections, graphing, and solving the equation to obtain calculated results (% Normal ADAMTS-13 Activity) for the controls and plasma samples.

Plasma samples with calculated ADAMTS-13 activities greater than 100% should be reported as >100% Normal ADAMTS-13 Activity.

REFERENCES

1. Collection, Transport and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays. Approved Guideline H21-A4 NCCLS, Volume 23, Number 35, December 2003
2. K. Kokame, M. Matsumoto, Y. Fujimura, and T. Miyata, *Blood,* **103**, 607 (2004).
3. K. Kokame, Y. Nobe, Y. Kokubo, A. Okayama, and T. Miyata, *Br. J. Haematol.*, **129**, 93 (2005).
4. Bernhard Lämmle and James N. George, *Seminars in Hematology*, **41**, 1, 1 (2004).
5. Lämmle B, Kremer Hovinga JA, Alberio L., *J Thromb Haemost,* **3**, 1663 (2005).

GTI®DIAGNOSTICS
Good science starts with people.™

20925 Crossroads Circle, Suite 200
Waukesha, WI 53186-4054 USA
(262) 754-1000 OR 1-800-233-1843

Code: 3224-s

Name: FRETS- VWF73

Storage: -20°C

Production: Synthetic Product

Asp- Arg- Glu- A2pr(Nma)- Ala- Pro- Asn- Leu- Val- Tyr- Met- Val- Thr- Gly- A2pr(Dnp)- Pro- Ala- Ser- Asp- Glu- Ile- Lys- Arg- Leu- Pro- Gly- Asp- Ile- Gln- Val- Val- Pro- Ile- Gly- Val- Gly- Pro- Asn- Ala- Asn- Val- Gln- Glu- Leu- Glu- Arg- Ile- Gly- Trp- Pro- Asn- Ala- Pro- Ile- Leu- Ile- Gln- Asp- Phe- Glu- Thr- Leu- Pro- Arg- Glu- Ala- Pro- Asp- Leu- Val- Leu- Gln- Arg

A2pr(Nma): N^{b}- [2- (Methylamino)benzoyl]- 2,3- diaminopropionic acid

(Trifluoroacetate Form)

(M.W. 8314.30) $C_{370}H_{583}N_{103}O_{113}S_1$

Fluorescence-Quenching Substrate for ADAMTS-13

References:

1. K. Kokame, M. Matsumoto, Y. Fujimura, and T. Miyata, *Blood*, **103**, 607 (2004). *(VWF73 Sequence)*
2. K. Kokame, Y. Nobe, Y. Kokubo, A. Okayama, and T. Miyata, *Br. J. Hematol.*, **129**, 93 (2005). *(FRETS-VWF73)*

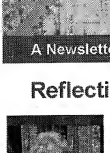
Package	Price(Yen)
Vial 0.1 mg	30,000

Back

A Newsletter from Peptides International

Volume 21, No. 1, 2008

Reflections on Twenty-Five Years . . .



Jackie B. Spatola

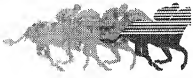
This year Peptides International will celebrate its twenty-fifth year of serving the peptides industry. Since the company's founding by her late husband, Dr. Arno F. Spatola, Jackie Spatola has played a key role in the operational aspects of the company and remains its principal owner and Chair of the Board. With her many years of experience, Jackie provides the following perspective on PI as it reaches this important milestone:

What would you say has changed the most about the business in the past 25 years?

Well, I would have to say that the emergence of PI as an in-house manufacturer of custom and catalog peptides has driven the greatest amount of change, both in terms of employees and facilities. Initially, the company assembled peptide synthesizers and distributed peptides that were all produced externally. We had just a few employees and a small amount of leased space. Today we have a full complement of research scientists, quality control specialists, order processors, and support staff in many different areas. We have our own laboratories, designed by our own employees, and a site that allows for further expansion. It has been a remarkable transition.

Are there any things that haven't changed?

PI has always had a family-like atmosphere and remains that way today. We are still proud to call Louisville, Kentucky our home. We have chosen to grow from within and have avoided some of the cultural changes that can occur with outside investment or ownership change. As a result the founding principals of the company – respect and treatment of others as we would want for ourselves, adherence to high ethical standards and a continued passion for quality continue to guide us each day.



Leading by Excellence for 25 Ye.

What do you believe are some of the greatest challenges the company has faced?

Of course, competition is much greater and more demanding now than it was when PI was one of just a very few peptide-focused companies. This has stimulated a continued effort to develop new products while carefully controlling costs and not in any way sacrificing quality. In the early years, as with many businesses, cash flow was sometimes a challenge. In later years we had to manage growth and look for investment capital that would still allow us to make our own decisions on the direction of the business. This was not always easy.

How do you see the company continuing to evolve?

We are very fortunate to be providing products used in so many exciting new areas of pharmaceutical research. Increasingly, this has led and will continue to lead us to leverage our resources through partnerships with others involved in scaled-up manufacturing or specialized applications. We are looking for new outlets for distributing our products internationally, as well as improving our current channels domestically. We expect that this could result in some exciting announcements later in this milestone year. And as change occurs, I want to assure our customers, vendors, friends and employees that we will make every effort to maintain the high standards that we have kept for the past twenty-five years.

Tumor-Targeting Peptides

RGD peptides have been utilized for their potential in cancer treatment and tumor imaging. In particular, *c*(RGDfK) has been used successfully by radiolabeling with different isotopes. ^{18}F labeling of a glycopeptide based on *c*(RGDfK) produced a conjugate that was successful in targeting integrin-positive M21 melanoma xenograft.[1] Others have used *c*(RGDyK) in the design of a clinically stable nano-device with superior binding to $\alpha_v\beta_3$ integrins or coupled the RGD peptide to liposomes to improve drug delivery to target cells.[2,3] Great progress has been made in the area of molecular imaging and tumor angiogenesis with the aide of cyclic RGD peptides. PI offers an extensive and growing line of these important peptides.

CODE	PRODUCT	QTY	US$
PCI-3661-PI	***cyclo* (Arg-Gly-Asp-D-Phe-Lys)** *c*(RGDfK)	1 mg 5 mg 25 mg	49 149 595
PCI-3687-PI	***cyclo* (Arg-Gly-Asp-D-Phe-Glu)** *c*(RGDfE)	1 mg 5 mg 25 mg	49 149 595
PCI-3662-PI	***cyclo* (Arg-Gly-Asp-D-Tyr-Lys)** *c*(RGDyK)	1 mg 5 mg 25 mg	49 149 595

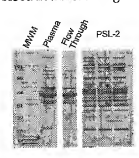
For a complete listing of RGD Peptides, visit *http://pepnet.com/products/rgdpeptides.pdf*

1. R. Haubner, *et al.*, *Cancer Res.*, **61**, 1781 (2001).
2. W. G. Lesnia, *et al.*, *Bioconjugate Chem.*, **18**, 1148 (2007).
3. R. Nallamothu, *et al.*, *AAPS PharmSciTech*, **7**, E1 (2006).

Peptides International, Inc.
PEPNET.COM
P.O. Box 24668
Louisville, KY 40224 USA
Phone: 502-266-8787
Fax: 502-267-1329
1-800-777-4779
E-mail: peptides@pepnet.com

Trace Protein Capture Using Peptide Libraries

ProSpectrum Libraries™ can be a highly useful tool for discovering trace components by simultaneously decreasing the concentrations of abundant proteins while enriching and capturing individual trace components on affinity ligands. ProSpectrum is a peptide library covalently linked to a bioaffinity resin that addresses current limitations in bioseparations. ProSpectrum is compatible with processing diverse samples, including whole blood in a broad range of formats and applications.



Evaluation of ProSpectrum Library PSL-2, Single LDS Elution

More proteins can be observed in the lanes treated with PSL-2 than can be detected from untreated plasma. Proteins detected in the flow through are similar to those in plasma, indicating that only a small fraction of each of the abundant proteins is captured by the library.

CODE	PRODUCT	QTY	USD
CPL-36325-PI	**ProSpectrum Library™ PSL-2** *Bioseparation Affinity Resin for Protein Diversity Capture*	100 mg 500 mg 1 g	385 1675 2495

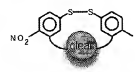
http://pepnet.com/ProSpectrumLibraries.html

ProSpectrum Libraries™ Featured in *Nature Protocols*!

"The Bead Blot: A Method for Selecting Small Molecule Ligands for Protein Capture and Purification." Julia Tait Lathrop, David Hammond, *Nature Protocols*, Nov 1, 2007

clear-ox™

CLEAR-OX™ is a highly effective polymer-supported reagent for the formation of disulfide-bonds.[1,2,3] Since the mechanism is based on peptide capture, sensitive residues such as Tyr, Trp, and Met are not affected, leading to increased purity and yield.



Single disulfide-bridged peptides with constrained amino acids have been successfully produced using CLEAR-OX with various ring sizes and lengths. Furthermore, oxidations are scalable with potential for recycling and automation using CLEAR-OX. Oxidations can be carried out immediately after cleavage with as low as two-fold excess of CLEAR-OX to obtain satisfactory yields. Most oxidations are completed within 1-2 hrs at an optimum pH < 7. While multiple bond formation is still under investigation, early reports indicate positive results.[4] CLEAR-OX is a superior choice for ease of use and when sensitive residues are present.

"I am convinced about the advantage of CLEAR-OX! I tried it on a peptide with an N-terminal Methionine. It oxidized for more than 50% with I_2/AcOH. Not with CLEAR-OX. Complete in 2 hours. Thanks!"

- Dr. Samuel Janssen, Amylin

CODE	PRODUCT	QTY	USD
RCO-1260-PI	**CLEAR-OX™** *Polymer-Supported Oxidant for Disulfide Bond Formation*	1 g 5 g	49 195

For more information, please visit: *http://pepnet.com/CLEAR-OX.pdf.*

1. K. Darlak, D.W. Long, A. Czerwinski, M. Darlak, F. Valenzuela, A.F. Spatola, and G. Barany. *J. Peptide Res.*, **63**, 303-312 (2004).
2. I. Annis, L. Chen, and G. Barany, *J. Am. Chem. Soc.*, **120**, 7226-7238 (1998).
3. CLEAR-OX™ US Patent Application 11/165,609 (June 23, 2005).
4. B.R. Green and G. Bulaj, *Protein Peptide Lett.*, **13**, 67-70 (2006).

Employee Spotlight

Peptides International is proud of the diversity of its employees who nonetheless share a common desire to offer our customers their absolute best in creating seriles and product wiine. This month we'd like to tell you a little about two of our newest, and maybe most eclectically schooled employees, Maia and Tim.

Maia Whang Arteel is responsible for synthesis and purification of peptides at PI, which must meet exacting specifications. Maia graduated from Bryn Mawr College with a B.A. in chemistry and a minor in mathematics, after which she undertook graduate studies in organic chemistry and German literature at UNC-Chapel Hill. Outside of PI, Maia enjoys yoga, contemporary literature, foreign and independent films, dining out, performing arts, and seeing the world with her son.

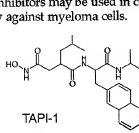
Timothy Morgan, one of PI's peptide chemists, went to the University of Louisville and received a B.S. in molecular biology. When not in the laboratory, Tim is finishing an M.A. in political science with a concentration in international relations. In his spare time, Tim enjoys reading about a wide range of subjects, playing guitar and violin, and keeping up with his two Great Danes.

Peptides International, Inc.
PEPNET.COM
P.O. Box 24658
Louisville, KY 40224 USA
Phone: 502-266-8787
Fax: 502-267-1329
1-800-777-4779
E-mail: peptides@pepnet.com

TAPI: TNF-Alpha Protease Inhibitor

The Matrix Metalloprotease (MMP) family participates in the regulation of cell development, wound healing, and diseases such as cancer and arthritis. TNF-Alpha Converting Enzyme or TACE is a member of the MMP family that is responsible for processing TNF by cleaving a 26 kDa proTNF to a soluble form.[1] A few recent studies have focused on TACE activity in cancer and its role in disease. In one study, researchers discovered that TACE downregulates TNF-related apoptosis-inducing ligand (TRAIL) receptor or death receptor 4 (DR4), decreasing the effect of TRAIL induced apoptosis on myeloma cells.[2] TACE inhibition could effectively upregulate DR4 and make myeloma cells more susceptible to TRAIL-mediated apoptosis. TACE inhibitors may be used in combination with TRAIL for therapy against myeloma cells.

TMEFF2 transmembrane protein is upregulated in 74% of primary prostate cancers with shedding induced by IL-1 and TNF.[3] **TAPI** treatment reduced TMEFF2 transmembrane protein shedding, lowering TMEFF2 induced cell proliferation. This study suggests that TACE and TMEFF2 could act as potential targets in the treatment for primary prostate cancer.



TAPI-1

TACE over expression was observed by Fabre-Lafay *et al.*, in breast tumor samples and over expression of active TACE has been shown by others to promote tumor growth by shedding pro-transforming growth factor-α.[4,5] In combination, these studies indicate TACE participates in cell proliferation and tumor growth, and suggest that TACE inhibitors may have some therapeutic benefit to patients with certain types of cancer. TAPI inhibitors are available for research purposes and for future development of compounds as potential pharmaceutical candidates through PI.

CODE	PRODUCT	QTY	USD
INH-3850-PI	**TAPI-0** HONHCOCH$_2$CH(CH$_2$CH(CH$_3$)$_2$) CO-Nal-Ala-NH$_2$	1 mg 5 mg	125 495
INH-3855-PI	**TAPI-1** HONHCOCH$_2$CH(CH$_2$CH(CH$_3$)$_2$) CO-Nal-Ala-NHCH$_2$CH$_2$NH$_2$	1 mg 5 mg	125 495
INH-3852-PI	**TAPI-2** HONHCOCH$_2$CH(CH$_2$CH(CH$_3$)$_2$) CO-t-Butyl-Gly-Ala-NHCH$_2$CH$_2$NH$_2$	1 mg 5 mg	125 495

Sold under license Research Corporation Technologies, Tucson
For more information, please visit: *http://pepnet.com/products/MMPQR.pdf*

1. K. Mohler, *et al., Nature*, **370**, 218 (1994).
2. K. Kagawa, *et al., Blood, (ASH Annual Meeting Abstracts)*, **110**, 244 (2007).
3. N. Ali and V. Knauper, *J. Biol. Chem.*, **282**, 373 (2007).
4. S. Fabre-Lafay, *et al., J. Biol. Chem.*, **280**, 19543 (2005).
5. M. Borrell-Pages, *et al., EMBO J.*, **22**, 1114 (2003).

Peptide Substrate for Blood-Related Diseases

New

Von Willebrand factor (vWF) is a multimeric glycoprotein that helps recruit platelets to injured vessel walls to promote healing and is secreted constitutively by endothelial cells and platelets. Promotion of platelet aggregation is dependent on the balanced distribution of large vWF multimers. ADAMTS-13 is a protease that helps regulate this process by cleaving larger VWF multimers into smaller forms in normal individuals. Functional deficiency of ADAMTS-13 causes accumulation of unusually large vWF multimers in the plasma which leads to continued platelet aggregation, depletion of platelets from the blood, and hemolysis. Disfunction of ADAMTS-13 causes blood-related diseases and conditions such as hemolytic anemia, thrombocytopenia, and thrombotic thrombocytopenic purpura (TTP).

A fluorescent quenching substrate and assay using **FRETS-VWF73** (*SFR-3224-s*) was initially developed by Kokame for diagnosis of TTP and thrombotic microangiopathies.[1,2] Research has accelerated in these areas leading to a reliable assay for detection of disease in patients with TTP. The assay has also furthered research into the role of auto antibodies in TTP, characterization of the substrate specificity, and the role of the protease in other blood-related diseases.[3-4] For instance, it has been used to demonstrate significant decrease in ADAMTS-13 activity in patients with acute myocardial infarction and acute ischemic stroke indicating a possible role of the protease in arterial thrombosis.[5] For the first time, the deregulation of ADAMTS-13 was shown to contribute in the pathogenesis of anthrax disease.[6] *B. antiracis* infection was shown to terminate normal regulation of vWF cleavage leading to increased hemorrhaging and thrombosis associated with anthrax toxicity. Deregulation occurs when bacterial virulence factors cleave and degrade vWF and ADAMTS-13. This fluorescent quenching substrate should continue to aide researchers in the understanding of blood related diseases and the mechanisms involved. FRETS-VWF73 substrate is offered by PI under license of the National Cardiovascular Center in Japan.

CODE	PRODUCT	QTY	USD
SFR-3224-s	**FRETS-VWF73** *Fluorescence-Quenching Substrate for ADAMTS-13*	0.1 mg vial	312

http://www.pepnet.com/products/fretsvwf73.pdf

1. K. Kokame, M. Matsumoto, Y. Fujimura, and T. Miyata, *Blood*, **03**, 607 (2004).
2. K. Kokame, Y. Nobe, Y. Kokubo, A. Okayama, and T. Miyata, *Br. J. Haematol.*, **129**, 93 (2005).
3. S.G. Shelat, P. Smith, J. Au, and X.L. Zheng, *J. Thromb. Haemost.*, **4**, 1707 (2006).
4. J. Au, P. Smith, S. Wang, P. Zhang, and X.L. Zheng, *J. Biol. Chem.*, **280**, 29428 (2005).
5. N. Dong, F. Liu, S.J. Master, and C. Ruan, *Blood*, **110**, 3987 (2007).
6. M.-C. Chung, T.C. Popova, S.C. Jorgenson, L. Dong, V. Chandrake, Charles L. Bailey, and S.G. Popov, *J. Biol. Chem.*, In press (2008).

Peptides International, Inc.
PEPNET.COM
P.O. Box 24658
Louisville, KY 40224 USA
Phone: 502-266-8787
Fax: 502-267-1329
1-800-777-4779
E-mail: peptides@pepnet.com

New Glycopeptide: APF from Interstitial Cystitis Patients

Antiproliferative factor (APF) (*CAR-24007-v*) is a low molecular weight, heat stable sialoglycopeptide that contains the transmembrane segment of Frizzled 8.1. APF is produced and secreted by bladder epithelial cells of patients with a condition known as interstitial cystitis (IC) which causes chronic pain due to ulcers, hemorrhaging, and thinning of the bladder epithelium.[1,2] APF contributes to the pathology of IC by inhibiting bladder cell proliferation, and was also shown to regulate proliferation of bladder carcinoma cells.[2,3] Recently, CKAP4/p63 was identified as a receptor for APF, but mediation of its activity remains unknown.[4]

APF's role as a negative regulator makes it a possible target for treatment of disease, and its unique presence in the urine of IC patients indicates it is a biomarker that may lead to a diagnostic tool for those suffering with IC. Chemists in the division of carbohydrate chemistry at the Peptide Institute achieved the total synthesis of this sialoglycopeptide, APF. In addition to other carbohydrate related products, APF is now available from PI to aid in IC research .

CODE	PRODUCT	QTY	USD
CAR-24007-v	**Antiproliferative Factor Sialoglycopeptide** **APF Sialoglycopeptide** ***Antiproliferative Factor from Interstitial Cystitis Patients***	50 µg vial	212

More informaton can be found at:
http://uuuu.pepnet.com/products/AntiproliferativeFactor_APF.pdf

1. S.K. Keay, *et al*, *Proc. Natl. Acad. Sci.U. S. A.*, **101**, 11803 (2004).
2. S.K. Keay, *et al*., *J. Urol.*, **64**, 2112 (2000).
3. S.K. Keay, *et al*., *J. Urol.*,**156**, 2073 (1996).
4. T.P. Conrads, *et al*., *J. Biol. Chem.*, **281**, 37836 (2006).

NEW Products of Interest:

PRODUCT	CODE
Protease-Activated Receptor (PAR) Peptides *http://pepnet.com/products/parpeptides.pdf*	
H-Tyr-Ala-Pro-Gly-Lys-Phe-NH_2	PAR-3933-PI
H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH	PAR-3934-PI
H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-NH_2	PAR-3935-PI
H-Ser-Phe-Leu-Leu-Arg-OH	PAR-3936-PI
H-Asn-Tyr-Pro-Gly-Lys-Phe-OH	PAR-3938-PI
H-Ser-Phe-Leu-Leu-OH	PAR-3941-PI
H-Ser-Phe-Leu-Leu-Arg-NH_2	PAR-3942-PI
H-Phe-Leu-Leu-Arg-Asn-OH	PAR-3944-PI
Enzyme Substrates	
Abz-Gly-Phe(NO$_2$)-Pro-OH	BPQ-3957-PI
Suc-Ala-Glu-Pro-Phe-pNA	SAP-3947-PI
[Sar^1]-Angiotensin II	SAN-3948-PI
[Sar^1]-Ang-Val-Tyr(I)-His-Pro-Phe-OH	

Please go to *http://pepnet.com/NewProducts2007.pdf* for further information.

2008 Derby Glass Promotion...

As in previous years, we're including a KY Derby commemorative glass with qualifying orders. These glasses are highly collectable and will be shipped - free to you - for any order over $500, *while quantities last during racing season. Limit one per laboratory please*

for the most up-to-date news ——• **PEPNET.com**

PEPTIDES INTERNATIONAL
P.O. Box 24658
Louisville, KY 40224
USA

Forwarding Service Requested

PRESORTED
STANDARD
U.S. Postage
PAID
PEPTIDES
INTERNATIONAL